Signalling cascades activated by the chemokine MCP-1

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SIGNALLING CASCADES ACTIVATED BY THE CHEMOKINE MCP-1

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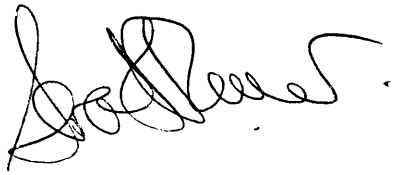

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ABSTRACT

The actions of the C-C chemokine, monocyte chemotactic peptide-1 (MCP-1) have been investigated on the human monocytic cell line THP-1, human embryonic kidney (HEK) 293 cells transfected with the high affinity MCP-1 receptors A and B and in human blood derived monocytes.

MCP-1 specifically bound to high affinity receptors on THP-1 cells and HEK 293 cells transfected with the type B receptor. MCP-1 bound to only a small population of HEK 293 cells transfected with the type A receptor.

MCP-1 also induced a dose-dependent increase in $[Ca^{2+}]_i$ in THP-1 cells, type B receptor transfectants and human monocytes, although the source of this intracellular Ca^{2+} varied. The Ca^{2+} chelator EGTA, the Ca^{2+} channel blocker Ni^{2+} and the surrogate Ca^{2+} ion Mn^{2+}, all demonstrated the requirement of extracellular Ca^{2+} in the MCP-1-induced responses in type B receptor transfected cells and monocytes. In contrast, THP-1 cells did not require extracellular calcium. Time-dependent increases in IP_3 were also detected in THP-1 cells and both the type A and type B receptor transfected cells, demonstrating the activation of PLC by MCP-1.

The novel signal transduction pathway, phosphatidylinositol 3-kinase (PI 3-kinase) was also activated by MCP-1 in a dose- and time-dependent manner in THP-1 cells and both the receptor transfected cells. However, by using two methods of studying PI 3-kinase activation, namely D-3 phospholipid accumulation or immunoprecipitation of the p85 subunit and *in vitro* lipid kinase assay, variations between the cell types were observed. MCP-1 induced the pertussis toxin sensitive accumulation of D-3 phospholipids in THP-1 cells, which was relatively insensitive to the PI 3-kinase inhibitor wortmannin. MCP-1 also induced an increase in a wortmannin sensitive, pertussis toxin insensitive PI 3-kinase activity in p85 immunoprecipitates. The type A receptor transfected cells only demonstrated MCP-1-induced D-3 phospholipid accumulation and the type B receptor transfected cells only demonstrated increased immunoprecipitated PI 3-kinase activity in response to MCP-1.

Three proteins, of molecular weight 120, 80 and 50kDa,were observed to be tyrosine phosphorylated in response to MCP-1 in the THP-1 cells and the type B receptor transfected cells. In addition, in THP-1 cells it was a distinct 55kDa phosphorylated protein which co-precipitated with the p85 subunit of PI 3-kinase.

Finally, MCP-1 induced an increase in chemotaxis in both human monocytes and THP-1 cells as well as adhesion molecule upregulation and superoxide release from human monocytes.

To Mum

With Love

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Abbreviations

β TG	β thromboglobulin
$(NH_4)_2$ HPO_4	*di*-ammoniumhydrogen orthophosphate
ANAP	anionic neutrophil activating peptide
ANOVA	analysis of variance
APS	ammonium persulphate
ATM	mutated gene in ataxia telangiectasia
ATP	adenosine trisphosphate
bcr	breakpoint cluster region
BSA	bovine serum albumin
Ca^{2+}	calcium
CC CKR	CC chemokine receptor
CCF 18	CC chemokine F18
CGD	chronic granulomatous disease
CHO K1	chinese hamster ovary K1 cells
CIF	calcium influx factor
Con A	concanavalin A
Cpk	C2 containing PI 3-kinase
CTAP-III	connective tissue activating peptide-III
DAG	diacylglycerol
DARC	Duffy antigen receptor for chemokines
DMEM	Dulbeccos modified eagles medium
DMSO	dimethyl sulphoxide
DNA-PK_{cs}	DNA-dependent protein kinase catalytic subunit
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	[ethylenebis(oxyethylenenitrilo)tetra-acetic] acid
EGTA-AM	EGTA-acetomethoxyester
ENA-78	epithelial cell-derived neutrophil-activating protein-78
ERK-2	extracellular signal regulated kinase-2
FACS	fluorescence activated cell sorter
FAD	flavin adenine dinucleotide
FAK	focal adhesion kinase
FCS	foetal calf serum

FITC	fluorescein isothiocyanate
FKBP12	FK506 binding protein 12
fMLP	formyl-methionyl-leucyl-phenylalanine
FRAP	FK506 binding protein rapamycin-associated protein
Fura-2/AM	fura-2/acetomethoxyester
GAP	GTPase-activating protein
GCF	granulocyte chemotactic factor
GCP-2	granulocyte chemotactic protein-2
GDP	guanosine diphosphate
GDPβS	guanosine 5'-*O*-(thio)diphosphate
GM-CSF	granulocyte/macrophage-colony stimulating factor
GTP	guanosine trisphosphate
GTPγS	guanosine 5' -3-*O*-(thio)triphosphate
HBSS	Hanks balanced salt solution
HCl	hydrochloric acid
HEK 293 cells	human embryonic kidney 293 cells
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HPLC	high performance liquid chromatography
HTRP	human *trp* homologue 1
I 309/TCA 3	T cell activation gene-3
ICAM-1, 2	intercellular adhesion molecule-1, 2
I_{CRAC}	calcium-release-activated calcium current
IFNγ, α	interferon γ, α
Ig superfamily	immunoglobulin superfamily
IgG, E	immunoglobulin G, E
IL-1, 3, 4, 5, 8	interleukin-1, 3, 4, 5, 8
IP-10	interferon inducible protein-10
IP_2	inositol (1,4) bisphosphate
IP_3 R	IP_3 receptor
IP_3	inositol (1,4,5) trisphosphate
IP_4	inositol (1,3,4,5) tetrakisphosphate
IRS-1	insulin receptor substrate-1
KOH	potassium hydroxide
LDCF	leukocyte derived chemotactic factor

LESTER	leukocyte expressed seven transmembrane domain receptor
LFA-1	leukocyte function antigen-1
LiCl	lithium chloride
LIX	LPS-induced C-X-C chemokine
LPS	lipopolysaccharide
LtB_4	leukotriene B_4
LtC_4	leukotriene C_4
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAP kinase	mitogen activated protein kinase
MCA	monocyte chemotactic activity
MCP-1, 2, 3, 4	monocyte chemotactic peptide-1, 2, 3, 4
M-CSF	macrophage-colony stimulating factor
MDNCF	monocyte derived neutrophil chemotactic factor
MEM	modified eagles medium
Mg^{2+}	magnesium
MGSA	melanocyte growth stimulating activity
Mig	monokine induced by gamma interferon
MIP-1α, 1β, 1γ, 2	macrophage inflammatory protein-1α, 1β, 1γ, 2
Mn^{2+}	manganese
MRP-1, 2	MIP related protein-1, 2
NaCl	sodium chloride
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NAF	neutrophil activating factor
NaOH	sodium chloride
NAP-2	neutrophil activating protein-2
NCF	neutrophil chemotactic factor
Ni^{2+}	nickel
norpA	no receptor potential A
NSI HIV-1	non-syncytium-inducing human immunodeficiency virus-1
PAF	platelet activating factor
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PDGF	platelet derived growth factor
PF 4	platelet factor 4
PHA	phytohemagglutinin
PI 3-kinase	phosphatidylinositol 3-kinase
PSF	polymorph stimulating factor
PtdC	phosphatidylcholine
PtdIns	phosphatidylinositol
PtdIns(3)P	phosphatidylinositol (3)-phosphate
PtdIns(3,4)P_2	phosphatidylinositol (3,4)-bisphosphate
PtdIns(3,4,5)P_3	phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(4,5)P_2	phosphatidylinositol (4,5) bisphosphate
PKC, B	protein kinase C, B
PLC, D, A_2	phospholipase C, D, A_2
PMA	phorbol 12-myristate 13-acetate
PMN cells	polymorphonuclear cells
PS	phosphatidylserine
PTK	protein tyrosine kinase
PVPF	polyvinylpyrolidine free
RAFT	rapamycin and FK506 binding protein 12 target
RANTES	regulated on activation normal T cell expressed and secreted
ROC	receptor operated channel
SD	standard deviation
SDF-1α, 1β	stromal cell derived factor-1α, 1β
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH2, 3 domains	src homology 2, 3 domains
sLex	sialyl Lewis X
SMC-CF	smooth muscle cell derived chemotactic factor
SMG	small GTP binding protein
SMOC	second messenger operated channel
SOD	superoxide dismutase
TBS	Tyrodes buffered saline
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethylethylenediamine

TLC	thin layer chromatography
TNF-α	tumour necrosis factor-α
TOR1, 2	target of rapamycin1, 2
Tris	Tris(hydroxymethyl)aminomethane
trp	transient receptor potential
TRPC	*trp* related protein 1
trpl	transient receptor potential-like
UTP	uridine triphosphate
VCAM-1	vascular adhesion molecule-1
VLA-4	very late antigen-4
VOC	voltage operated channel
Vps 15, 34	vacuolar protein sorting mutant

Single letter amino acid code

A	Ala
C	Cys
D	Asp
E	Glu
F	Phe
G	Gly
H	His
I	Ile
K	Lys
L	Leu
M	Met
N	Asn
P	Pro
Q	Gln
R	Arg
S	Ser
T	Thr
V	Val
W	Trp
Y	Tyr

List of figures

		Page
Section 1		
Figure 1.1	Diagram to represent leukocyte transmigration	4
Figure 1.2	Amino acid sequence and structure of MCP-1	18
Figure 1.3	Activation of PLC	25
Figure 1.4	Pathway to show the calcium mobilisation and calcium influx interrelationship	33
Figure 1.5	The synthesis of D-3 phosphorylated phosphoinositides	36
Figure 1.6	The activation of PI 3-kinase isoforms	45
Section 2		
Figure 2.1	A typical standard curve for the measurement of IP_3	75
Figure 2.2	An example of a trace showing the retention times of the [^{3}H]-PtdIns and [^{3}H]-PtdIns(4,5)P_2 standards used in the analysis of PI 3-kinase products	77
Figure 2.3	Example of an SDS-PAGE gel to show coupling of the p85-antibody to the protein G sepharose beads	81
Figure 2.4	Diagram to show the reaction which takes place in the *in vitro* lipid kinase assay	82
Figure 2.5	Diagram of the Boyden Chamber set-up	87
Figure 2.6	A typical standard curve for the measurement of hydrogen peroxide generation using scopoletin	90

Figure 2.7 A typical standard curve for the measurement of protein content in the samples used in the ferricytochrome C assay 92

Section 3

Figure 3.1 [^{125}I]-MCP-1 binding to THP-1 cells 98

Figure 3.2 [^{125}I]-MCP-1 binding to CC CKR 2A and 2B transfected cells 99

Figure 3.3 Binding of biotinylated MCP-1 to THP-1 cells 100

Figure 3.4 Binding of biotinylated MCP-1 to CC CKR 2A and CC CKR 2B transfected cells 101

Figure 3.5 Binding of biotinylated MCP-1 to untransfected HEK 293 cells 102

Section 4

Figure 4.1 Dose-dependent effects of MCP-1 on the $[Ca^{2+}]_i$ response in THP-1 cells 104

Figure 4.2 Desensitisation effects of repeated chemokine stimulation 105

Figure 4.3 Desensitisation effects of repeated chemokine stimulation 106

Figure 4.4 Dose-dependent effects of ATP on the $[Ca^{2+}]_i$ response in THP-1 cells 109

Figure 4.5 Effects of calcium influx inhibitors on the MCP-1-induced increase in $[Ca^{2+}]_i$ 110

Figure 4.6 Effects of calcium influx inhibitors on the ATP-induced increase in $[Ca^{2+}]_i$ 112

Figure 4.7	Mn^{2+} influx in THP-1 cells in response to MCP-1 and ATP	113
Figure 4.8	Role of phospholipase C in the MCP-1-induced calcium response	115
Figure 4.9	$[Ca^{2+}]_i$ and IP_3 levels in THP-1 cells following 12.5nM MCP-1 stimulation	116
Figure 4.10	Role of PKC in the MCP-1-induced increase in $[Ca^{2+}]_i$	117
Figure 4.11	Role of PLC and PKC in the ATP-induced rise in $[Ca^{2+}]_i$	119
Figure 4.12	Effects of pertussis toxin on the MCP-1-induced rise in $[Ca^{2+}]_i$	120
Figure 4.13	Effects of pertussis toxin on the ATP-induced rise in $[Ca^{2+}]_i$	121
Figure 4.14	Effects of UTP on the $[Ca^{2+}]_i$ in THP-1 cells	122
Figure 4.15	Effects of MCP-1, related chemokines and EGF on the $[Ca^{2+}]_i$ elevation in CC CKR 2A transfected cells	124
Figure 4.16	Dose-dependent effects of MCP-1 and related chemokines on the $[Ca^{2+}]_i$ in CC CKR 2B transfected cells	125
Figure 4.17	$[Ca^{2+}]_i$ responses in untransfected HEK 293 cells in response to MCP-1 and EGF	126
Figure 4.18	MCP-1-induced increase in $[Ca^{2+}]_i$ in human blood-derived monocytes	128
Figure 4.19	Effects of EGTA on the MCP-1-induced increase in $[Ca^{2+}]_i$ in CC CKR 2B transfected cells	129

Figure 4.20 Mn^{2+} influx in CC CKR 2B transfected cells in response to MCP-1 130

Figure 4.21 Effects of EGTA and calcium readdition on the MCP-1-induced $[Ca^{2+}]_i$ changes in human monocytes 131

Figure 4.22 Effects of PLC inhibition on the MCP-1-induced increase in $[Ca^{2+}]_i$ in CC CKR 2B transfected cells and human monocytes 133

Figure 4.23 IP_3 levels in HEK 293 cells transfected with the CC CKR 2A, the CC CKR 2B and untransfected cells following 12.5nM MCP-1 stimulation 134

Figure 2.24 Effects of pertussis toxin on the MCP-1-induced increase in $[Ca^{2+}]_i$ in CC CKR 2B transfected cells 135

Section 5

Figure 5.1 Typical HPLC traces obtained from unstimulated cells and from cells stimulated with 180nM MCP-1 for 30 seconds following phospholipid extraction and deacylation 140

Figure 5.2 Timecourse of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 changes in THP-1 cells in response to 180nM MCP-1 stimulation 141

Figure 5.3 Concentration dependent changes in PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in THP-1 cells following 30 second incubations with various concentrations of MCP-1 143

Figure 5.4 Effects of pertussis toxin and wortmannin on the MCP-1-induced changes in PtdIns(3,4,5)P_3 in THP-1 cells 144

Figure 5.5 *In vitro* lipid kinase assay of THP-1 cells stimulated for various times with MCP-1 146

Figure 5.6 *In vitro* lipid kinase assay of THP-1 cells stimulated with various concentrations of MCP-1 147

Figure 5.7 *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells in the presence or absence of various concentrations of wortmannin 148

Figure 5.8 *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells in the presence or absence of various concentrations of pertussis toxin 149

Figure 5.9 Changes in PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in CC CKR 2A transfected cells 151

Figure 5.10 Changes in PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in CC CKR 2B transfected cells 153

Figure 5.11 Changes in PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in untransfected HEK 293 cells 154

Figure 5.12 *In vitro* lipid kinase assay of CC CKR 2A cells stimulated with various concentrations of MCP-1 or 100ng/ml EGF 155

Figure 5.13 *In vitro* lipid kinase assay of CC CKR 2B cells stimulated with various concentrations of MCP-1 or 100ng/ml EGF 158

Figure 5.14 Tyrosine phosphorylation of proteins following MCP-1 stimulation of THP-1 cells 159

Figure 5.15 Tyrosine phosphorylated proteins associated with the p85 subunit of PI 3-kinase in THP-1 cells 160

Figure 5.16 Tyrosine phosphorylation of proteins following MCP-1 stimulation of CC CKR 2A and 2B transfected cells 163

Figure 5.17 Effects of MCP-1 on p70 S6 kinase phosphorylation in THP-1 cells 164

Figure 5.18 [^{3}H]-thymidine incorporation into CC CKR 2A transfectants, CC CKR 2B transfectants and untransfected HEK 293 cells 166

Figure 5.19 [^{3}H]-thymidine incorporation into CC CKR 2A transfectants, CC CKR 2B transfectants and untransfected HEK 293 cells in response to MCP-1 167

Section 6

Figure 6.1 Chemotactic response to MCP-1 in human blood-derived monocytes and THP-1 cells 171

Figure 6.2 Effects of MCP-1 on CD11b and CD11c expression on human monocytes 173

Figure 6.3 Effects of MCP-1 on VLA-4 expression on human monocytes 175

Figure 6.4 Upregulation of CD11b and CD11c by MCP-1 on human monocytes in the absence or presence of 100nM wortmannin 176

Figure 6.5 Generation of hydrogen peroxide in purified human monocytes following stimulation by MCP-1, C5a, fMLP and PMA 178

Figure 6.6 Generation of superoxide by purified human monocytes following stimulation by MCP-1, C5a, fMLP and PMA 179

Figure 6.7 Effects of MCP-1 on the upregulation of CD23 on human monocytes 181

Section 8

Figure 8.1 Schematic diagram to represent the proposed pathways activated by MCP-1 223

Figure 8.2 Schematic diagram to show the proposed roles for the signalling pathways activated by MCP-1 224

List of tables

		Page
Section 1		
Table 1.1	Receptors involved in leukocyte adhesion to the endothelium	3
Table 1.2	Members of the human chemokine family	10-11
Table 1.3	Members of the chemokine receptor family	14
Table 1.4	PLC isoforms identified in mammalian tissues	23
Table 1.5	PKC isoforms found in mammalian tissues	34
Table 1.6	PI 3-kinase family members	43
Table 1.7	Responses observed to be potently inhibited by wortmannin	53
Section 2		
Table 2.1	Primary and secondary antibodies used for the development of tyrosine phosphorylation and p70 S6 kinase phosphorylation Western blots	85
Section 4		
Table 4.1	Effects of repeated chemokine addition on the $[Ca^{2+}]_i$ in THP-1 cells	108
Section 8		
Table 8.1	Summary of MCP-1-induced responses and the optimal concentrations and times	227

Appendix 5

Table A5 Recipes for various percentage SDS-PAGE gels 235

Table of contents

Page

Section 1 : Introduction

1.1 Background 1

1.2 Adhesion molecules 2

1.3 Chemokines 7

1.3.1 C-X-C chemokines 8

1.3.2 C-C chemokines 8

1.3.3 Chemokine receptors 9

1.3.3.1 C-X-C chemokines 9

1.3.3.2 C-C chemokine receptors 12

1.3.3.3 Other chemokine receptors 13

1.3.4 Role of chemokines in disease 15

1.4 Role of adhesion molecules and chemokines in leukocyte migration 16

1.5 Monocyte chemotactic peptide-1 17

1.5.1 Identification of MCP-1 17

1.5.2 MCP-1 structure 18

1.5.3 Biological effects of MCP-1 19

1.6 Signalling pathways 21

1.6.1 Phospholipase C 22

1.6.1.1 PLC subtypes and their activation 23

1.6.1.1.1 PLC β activation 23

1.6.1.1.2 PLC γ activation 24

1.6.1.1.3 PLC δ activation 24

1.6.1.1 IP_3 receptors and their function 26

1.6.1.2 Capacitative calcium entry 28

1.6.1.3 Activation of PKC isoforms by DAG 32

1.6.2 Phosphatidylinositol 3-kinase 34

1.6.2.1 Formation of D-3 PtdIns lipids 34

1.6.2.2 PI 3-kinase family 37

1.6.2.2.1 PI 3-kinase identification 37

1.6.2.2.2 PTK/SH2-coupled PI 3-kinase structure 37

1.6.2.2.3 G-protein-coupled PI 3-kinase 39

1.6.2.2.4 PtdIns-specific PI 3-kinase 40

1.6.2.2.5 PI 3-kinase in yeast 40

1.6.2.2.6 Other PI 3-kinase activities 41

1.6.2.3 PI 3-kinase coupling and activation 41

1.6.2.3.1 PTK/SH2-coupled PI 3-kinase 41

1.6.2.3.2 PI 3-kinase γ 46

1.6.2.4 Inhibitors of PI 3-kinase 47

1.6.2.4.1 Wortmannin 47

1.6.2.4.2 Other inhibitors of PI 3-kinase 48

1.6.2.4.3 Alternative approaches to studying PI 3-kinase 49

1.6.2.4.4 Downstream effectors of PI 3-kinase 50

1.7 Monocyte functional changes 54

1.8 Aims of study 57

Section 2 : Materials and Methods

2.1 Materials 58

2.2 Methods 64

2.2.1 Cell culture 64

2.2.1.1 THP-1 cells 64

2.2.1.2 HEK 293 cells 64

2.2.1.3 Monocyte preparation 64

2.2.2 Radioligand binding 66

2.2.3 Binding by FACS analysis 68

2.2.3.1 Assay procedure 68

2.2.4 Measurement of $[Ca^{2+}]_i$ 69

2.2.4.1 $[Ca^{2+}]_i$ measurements in a cell population 70

2.2.4.1.1 Loading cells with fura-2/AM 70

2.2.4.1.2 $[Ca^{2+}]_i$ measurement 70

2.2.4.1.3 Fluorimeter calibration 71

2.2.4.2 $[Ca^{2+}]_i$ measurements on a small population of adherent cells 72

2.2.5 Inositol (1,4,5) trisphosphate (IP_3) measurements 72

2.2.5.1 Sample preparation 73

2.2.5.2 Detection of IP_3 73

2.2.6 PI 3-kinase activity 74

2.2.6.1 Accumulation of D-3 phosphatidylinositol lipids in intact cells 74

2.2.6.1.1 Sample preparation 74

2.2.6.1.2 HPLC analysis of samples 76

2.2.6.2 Immunoprecipitation and *in vitro* lipid kinase assay 78

2.2.6.2.1 Coupling the antibody to protein G sepharose beads 78

2.2.6.2.2 Immunoprecipitation of the PI 3-kinase 79

2.2.6.2.3 *In vitro* lipid kinase assay 80

2.2.7 Detection of tyrosine phosphorylated proteins 82

2.2.7.1 Sample preparation 82

2.2.8 Phosphorylation of p70 S6 kinase 83

2.2.8.1 Sample preparation 83

2.2.9 SDS-PAGE and Western blotting 84

2.2.9.1 Separation of proteins 84

2.2.9.2 Development of the blot 84

2.2.10 Proliferation assay 85

2.2.11 Chemotaxis 86

2.2.12 Adhesion molecule expression 88

2.2.13 Measurement of superoxide release from monocytes 88

2.2.13.1 Scopoletin assay 89

2.2.13.2 Ferricytochrome C assay 89

2.2.13.2.1 Protein assay 91

2.2.14 CD23 upregulation on human monocytes 93

2.2.15 Statistical analysis 93

Section 3 : MCP-1 binding studies

3.1 [^{125}I]-MCP-1 binding 94

3.1.1 THP-1 cells 94

3.1.2 CC CKR 2A and 2B transfected cells 94

3.2 Biotinylated MCP-1 binding 95

3.2.1 THP-1 cells 95

3.2.2 CC CKR 2A and 2B transfected cells 96

3.3 Summary 96

Section 4 : Characterisation of the MCP-1-induced rise in $[Ca^{2+}]_i$

4.1 Measurement of $[Ca^{2+}]_i$ in THP-1 cells 103

4.1.1 Elevation of $[Ca^{2+}]_i$ by MCP-1 103

4.1.2 Desensitisation of the MCP-1-induced calcium response 103

4.1.3 Identification of the source on calcium responsible for the MCP-1-induced $[Ca^{2+}]_i$ elevation 107

4.1.3.1 Role of calcium influx in the MCP-1-induced rise in $[Ca^{2+}]_i$ 107

4.1.3.2 Involvement of PLC in the MCP-1-induced elevation in $[Ca^{2+}]_i$ 114

4.1.3.3 Role of PKC in the MCP-1-induced elevation of $[Ca^{2+}]_i$ 114

4.1.3.4 Coupling of the MCP-1-induced elevation of $[Ca^{2+}]_i$ in THP-1 cells to G-proteins 118

4.2 Detection of the MCP-1-induced $[Ca^{2+}]_i$ elevations in CC CKR 2A and 2B transfectants and human monocytes 123

4.2.1 Comparison of MCP-1-induced calcium responses 123

4.2.2 Comparison of the source of the MCP-1-induced $[Ca^{2+}]_i$ elevation in CC CKR 2B transfected cell and human monocytes 127

4.2.2.1 Role for calcium influx in the increase in $[Ca^{2+}]_i$ 127

4.2.2.2 Determination of the role of PLC in the MCP-1-induced calcium responses 132

4.2.2.3 Coupling of the MCP-1-induced $[Ca^{2+}]_i$ response in CC CKR 2B transfectants to G-proteins 136

4.3 Summary 136

Section 5 : MCP-1 induced activation of PI 3-kinase

5.1 PI 3-kinase activation by MCP-1 in THP-1 cells 139

5.1.1 Accumulation of D-3 phosphatidylinositol lipids 139

5.1.1.1 Identification of the PI 3-kinase sub-type activated by MCP-1 142

5.1.2 Determination of *in vitro* lipid kinase activity after MCP-1 stimulation 142

5.1.2.1 Further characterisation of the immuno-precipitated PI 3-kinase activity stimulated by MCP-1 145

5.2 MCP-1-induced activation of PI 3-kinase in CC CKR 2A and 2B transfected cells 150

5.2.1 MCP-1-induced accumulation of D-3 phosphatidyl-inositol lipids 150

5.2.2 Immunoprecipitation of MCP-1-induced PI 3-kinase in CC CKR 2A and 2B transfectants 152

5.3 Determination of protein tyrosine kinase coupling to MCP-1 receptors in THP-1 cells and CC CKR 2A and 2B transfectants 156

5.4 Determination of potential downstream effectors of PI 3-kinase activated by MCP-1 162

5.5 Role of MCP-1 in the proliferation rate of the CC CKR 2A and 2B transfected cells 165

5.6 Summary 168

Section 6 : Functional responses activated by MCP-1 in human monocytes and monocytic cell lines

6.1 MCP-1-induced chemotaxis in human monocytes and THP-1 cells 170

6.2 Upregulation of various adhesion molecules on human monocytes following MCP-1 stimulation 172

6.3 Superoxide generation in response to MCP-1 stimulation of human monocytes 177

6.4 Low affinity IgE receptor expression on human monocytes following MCP-1 stimulation 180

6.5 Summary 182

Section 7 : Discussion

7.1 The binding of MCP-1 to its receptors 183

7.2 Generation of calcium transients in response to MCP-1 187

7.2.1 MCP-1 induces a rise in $[Ca^{2+}]_i$ in THP-1 cells and human monocytes 187

7.2.2 Disparate calcium signals generated by MCP-1 in the CC CKR 2A and 2B transfected cells 193

7.2.3 Role for PLC and calcium in monocyte activation 196

7.3 MCP-1-induced activation of PI 3-kinase 197

7.3.1 MCP-1 activates at least two PI 3-kinase isotypes in THP-1 cells 197

7.3.2 Disparate PI 3-kinase activation by MCP-1 stimulation of CC CKR 2A and 2B receptor transfected cells 203

7.3.3 MCP-1 induces the tyrosine phosphorylation of proteins in THP-1 cells and CC CKR 2B transfectants 206

7.3.4 Activation of potential downstream effectors of PI 3-kinase 212

7.3.5 Role for PI 3-kinase and tyrosine phosphorylation in monocyte activation 213

7.5 MCP-1-induced functional responses in human monocytes and THP-1 cells 215

Section 8 : Conclusions

8.1 General conclusions 220

8.2 Proposed pathways activated by MCP-1 221

8.3 Future directions 228

Appendices 231

Bibliography 237

Publications 287

SECTION 1 : INTRODUCTION

1.1 Background

The classical signs of inflammation (redness, heat, swelling and pain) were reported as early as the second century when Celsius first described acute inflammation. These signs were further investigated in the eighteenth century by Hunter who characterised the four phases proposed by Celsius. He recognised that the redness and heat were due to increased blood flow following vasodilatation and the formation of new blood vessels (Hunter, 1794). He also suggested that the swelling was due to plasma exudation and that the pain was caused by hyperalgesia. Further advances were made by Cohnheim (Cohnheim, 1882). Using microscopic techniques on frog blood vessels, he traced the migration of white cells from the vasculature into the tissues. He proposed that it was this initial humoral response, arising from vascular changes due to a given stimulus, that was the basis of inflammation. In contrast, Metchnikoff recognised the phagocytic role of cells in the removal of microbial infection and proposed a cellular basis to the inflammatory response (Metchnikoff, 1893). It is now clear that both the humoral and cellular theories are very closely linked.

The inflammatory response involves the infiltration of circulating blood cells into the site of infection. Their primary role is to eliminate foreign particles, whether in the form of bacterial, viral or parasitic organisms. The roles played by the infiltrating cells are very different. Neutrophils and monocytes are involved in phagocytosis, basophils with the release of inflammatory mediators such as histamine and eosinophils in the destruction of larger parasitic infections by degranulation and release of toxic substances. Lymphocytes play an important role in the production of antibodies and the acquired immune response.

1.2 Adhesion molecules

Although it was Cohnheim who first observed that before leukocytes migrated into a tissue they engaged in an adhesion interaction with endothelial cells, it has only been over the last ten years that a number of ligand-counterligand molecules have been identified on leukocytes and endothelial cells. It is these adhesion molecules which provide the basis for leukocyte-endothelial cell adhesion (Butcher, 1991) (Springer, 1994) (Carlos & Harlan, 1994).

There are three main structural classes of adhesion molecules which are found on leukocytes and endothelial cells and these are summarised in table 1.1. The integrins are found on leukocytes, the Ig superfamily members on endothelial cells and the selectins on both cell types. It has become clear that there are three main steps involved in the transmigration of leukocytes into the site of inflammation and these are summarised in figure 1.1.

The first step involves the rolling of leukocytes along the surface of the endothelium and this can be recreated *in vitro* under conditions of shear stress using cultured endothelial cells (Lawrence *et al.* 1990). This initial rolling is mediated by L-selectin on leukocytes and P- and E- selectin on the endothelial layer (Lawrence & Springer, 1991). Interestingly, L-selectin is shed upon leukocyte activation and if the leukocytes becomes activated before their initial contact with the endothelium then they lose their ability to adhere and transmigrate (Kishimoto *et al.* 1989).

The second adhesion step involves the integrins and their counter-receptors the Ig superfamily. This second adhesion step cannot take place under conditions of shear stress if the preceding selectin-mediated step does not occur (Lawrence & Springer,

Table 1.1 - Receptors involved in leukocyte adhesion to the endothelium. The integrins are upregulated on leukocytes in response to various stimuli. Expression of the Ig superfamily on endothelial cells increases upon stimulation by pro-inflammatory stimuli such as IL-1 and TNF (except ICAM-2 which is constitutively expressed) as are the endothelial selectins. L-selectin is rapidly shed from activated leukocytes. (Modified from Wardlaw 1990 and Zimmerman *et al* 1992).

Leukocyte receptor	Adhesion molecule class	Expressed by	Endothelial cell receptor	Adhesion molecule class
VLA-4	β_1 integrin	Lymphocytes, monocytes	VCAM-1	Ig superfamily
LFA-1 (CD11a/CD18)	β_2 integrin	All leukocytes	ICAM-1, ICAM-2	
MAC-1 (CD11b/CD18)			ICAM-1	
p150,95 (CD11c/CD18)			Not known	-
L-selectin	Selectins	PMN's, monocytes, eosinophils, lymphocyte subsets	E-selectin, P-selectin, GlyCAM-1, CD34, MAdCAM-1	Selectins
P-selectin glycoprotein ligand (PSGL)-1		PMN's, monocytes, lymphocyte subpopulations, NK cells	P-selectin	

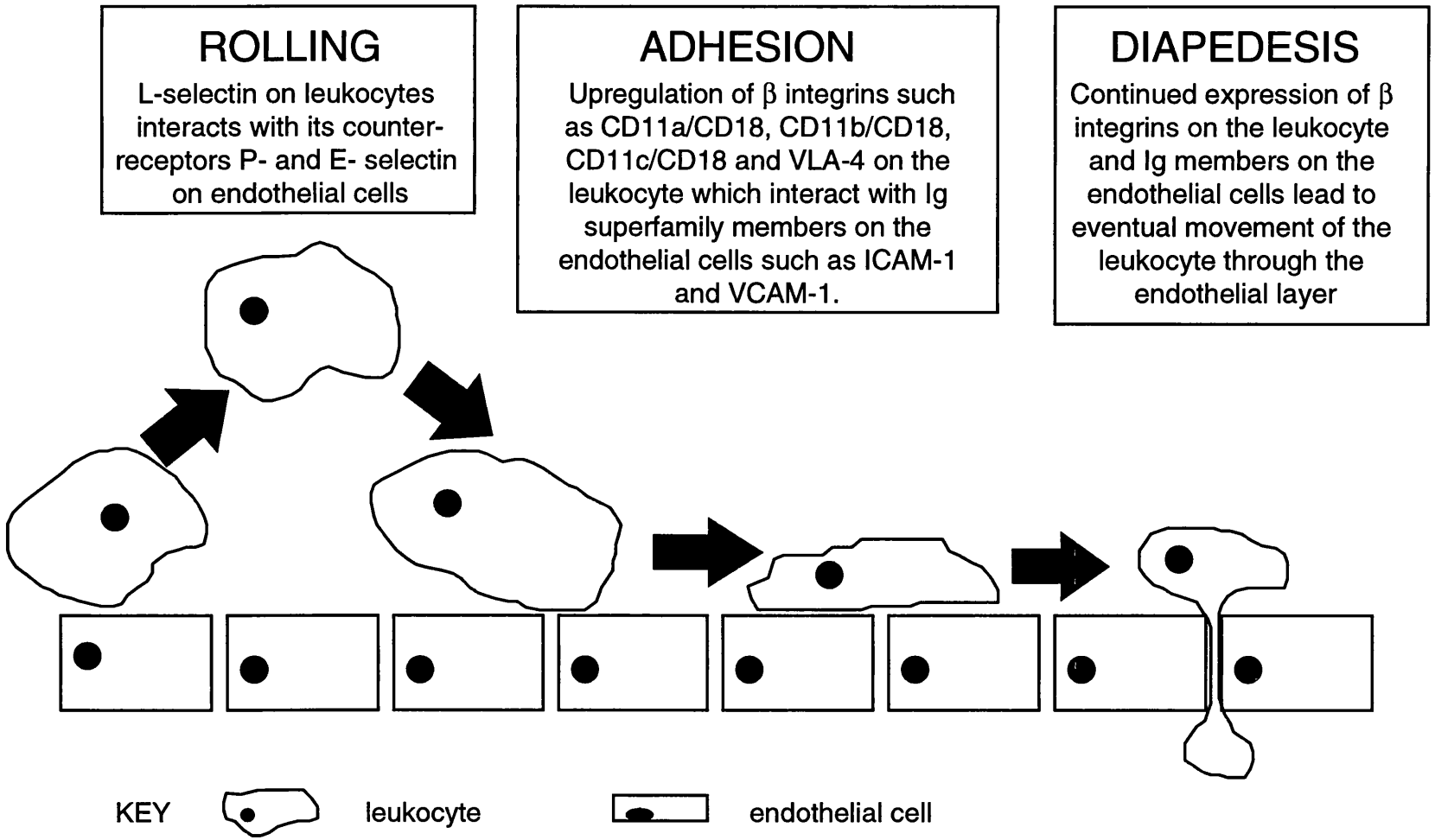


Figure 1.1 - Diagram to represent leukocyte transmigration. There are three stages involved in the infiltration of leukocytes into the site of inflammation. These three stages are characterised by the expression of various adhesion molecules. The chemokines play an important role in the upregulation of integrins for the firm adhesion of leukocytes as well as providing a chemotactic concentration gradient down which they can migrate.

1991). It allows firm adhesion of the leukocytes to the endothelial cells which is crucial for emigration and which is not provided by the selectins. The use of monoclonal antibodies has provided vital evidence for the interaction between various members of the adhesion molecule families on leukocytes and endothelial cells. It is mainly CD11b/CD18 that has been found to be of importance in the firm adhesion of leukocytes (Luscinskas *et al.* 1991) (Meerschaert & Furie, 1995). More recently, VLA-4 has also been shown to play a part in this firm adhesion (Meerschaert & Furie, 1994).

It is the continued expression of the integrins and Ig superfamily members that leads to the eventual transmigration of the leukocytes into the extravascular space. The Ig superfamily members are expressed at the intercellular junctions which direct the cells through the endothelial layer.

Thus, the movement of leukocytes from the peripheral vasculature into a site of inflammation involves the expression of these adhesion molecules on both leukocytes and endothelial cells. Although the expression of these molecules is very similar in all leukocytes, the arrival of leukocytes into a site of inflammation is kinetically different. For example; 1) there is a characteristic absence of monocytes in acute inflammatory responses but higher numbers are observed in chronic inflammation and 2) the chronic responses characterised by large numbers of monocytes are quite often preceded by a neutrophil mediated acute phase. This is particularly important considering that these cells perform different roles in an inflammatory site and thus, neutrophils appear more important in acute inflammation and monocytes in chronic inflammation. Some of the well characterised chemotactic factors such as C5a, leukotriene B_4 (LtB_4) and formyl methionyl leucyl phenylalanine (fMLP) are potent inducers of both monocyte and neutrophil chemotaxis but they cannot explain the

differential appearance of these leukocytes as they do not show any leukocyte specificity.

There are a number of possible explanations for these differences. Firstly, it could simply be a question of relative leukocyte numbers. Granulocytes make up about 80% of the circulating blood leukocytes and it may be that the infiltration of monocytes is masked by the neutrophils. Secondly, the positioning of the circulating cells may be important. Neutrophils marginate very close to the endothelial layer under normal conditions, unlike monocytes. Therefore, the delay in monocyte infiltration may be due to the prolonged time taken for the monocytes to interact with the endothelium. Thirdly, the presence of leukocyte specific factors which attract distinct leukocyte subsets may explain the disparate appearance of these leukocyte subtypes.

Concerning the third point, a breakthrough was made in 1987, when a neutrophil specific chemoattractant was isolated from lipopolysaccharide (LPS)-stimulated human monocyte-conditioned media (Yoshimura *et al.* 1987). This monocyte-derived neutrophil chemotactic factor (MDNCF) was isolated by a number of groups and given various names including neutrophil activating factor (NAF) (Baggiolini *et al.* 1989), granulocyte chemotactic factor (GCF) (Van Damme *et al.* 1988), neutrophil chemotactic factor (NCF) (Strieter *et al.* 1989) and polymorph stimulating factor (PSF) (Watson *et al.* 1989a) (Watson *et al.* 1988). All these groups had discovered a peptide of approximately 72 amino acids in length containing four cysteine residues. At a symposium entitled 'Novel neutrophil stimulating peptides', the decision was made to call this peptide interleukin-8 (IL-8) (Westwick *et al.* 1989).

Examination of the amino acid sequence of IL-8 led to the discovery of a group of proteins with conserved sequences which had been first identified as early as 1977 (Deuel *et al.* 1977). The increasing number of these chemotactic cytokines (Lindley *et*

al. 1993) and the discovery of two separate sub-families led to the term chemokines being adopted for all the conserved peptides.

1.3 Chemokines

All the members of the chemokine family have molecular weights of between 8-10kDa and they are basic heparin binding polypeptides. They are characterised by the presence of four conserved cysteine residues (review (Oppenheim *et al.* 1991)). There are two main subfamilies which can be distinguished by the position of the first two of the four cysteine residues. The C-X-C group, of which IL-8 is the best known member, possess an amino acid which separates the first two cysteine residues. In the C-C group, the two cysteine residues are juxtapositioned. Members of this group include monocyte chemotactic peptide-1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α and -1β.

The two chemokine sub-families differ both in their cell specificity and their chromosomal location. The C-X-C chemokines activate neutrophils and lymphocytes but not monocytes and their genes are co-localised on chromosome 4. The C-C chemokines activate monocytes, lymphocytes, basophils and eosinophils but not neutrophils and their genes are clustered on chromosome 17.

Very recently, a third chemokine subfamily, the C chemokines have been identified (Kelner *et al.* 1994). To date, only one member has been isolated and it has been named lymphotactin, due to its chemotactic properties for T lymphocytes. It only possesses the second and the fourth cysteine residue and its gene is positioned on chromosome 1.

A full listing of the human C-X-C, C-C and C chemokines is shown in table 1.2, and is current up to September 1996. The chemokines represented in bold in the table, indicate those which have been discovered in the last three years, that is during my research project.

1.3.1 C-X-C chemokines

The C-X-C chemokines are known to be released from a number of different cells in response to pro-inflammatory agents such as LPS, interleukin-1α (IL-1α), tumour necrosis factor α (TNFα) and phytohemagglutinin (PHA). These cells include monocytes (Yoshimura *et al.* 1987) (Haskill *et al.* 1990), neutrophils (Takahashi *et al.* 1993), epithelial cells (Cromwell *et al.* 1992), endothelial cells (Sica *et al.* 1990). fibroblasts (Golds *et al.* 1989) (Watson *et al.* 1989b) mesangial cells (Brown *et al.* 1991) and microvascular endothelial cells (Brown *et al.* 1994).

The C-X-C chemokines were originally reported as specific neutrophil activators with IL-8 inducing neutrophil chemotaxis (Schroeder & Christophers, 1992), degranulation and lysosomal enzyme release (Thelen *et al.* 1988), increased release of LtB_4 (Smith *et al.* 1992) and increased adherence to unstimulated endothelial cells (Carveth *et al.* 1989). More recently, IL-8 has also been observed to induce chemotaxis and the release of histamine from basophils (Krieger *et al.* 1992) as well as attract particular sub-sets of T lymphocytes (Larsen *et al.* 1989) (Bacon *et al.* 1989).

1.3.2 C-C chemokines

The C-C chemokines are produced by a number of different inflammatory and non-inflammatory cells including monocytes (Yoshimura *et al.* 1989b) (Chang *et al.* 1989), endothelial cells (Antoniades *et al.* 1992) (Brown *et al.* 1992) epithelial cells (Standiford *et al.* 1991) and fibroblasts (Bedard *et al.* 1993) (Watson *et al.* 1995). Their production is in response to various pro-inflammatory stimuli such as IL-1α,

TNFα and LPS. The C-C chemokines are potent activators of monocytes, eosinophils, lymphocytes and basophils, stimulating responses such as chemotaxis, histamine release, intracellular calcium rise and respiratory burst.

1.3.3 Chemokine receptors

A full listing of all the chemokine receptors identified to date is given in table 1.3. The receptors in the shaded boxes indicate those which have been identified in the last three years and demonstrate that the chemokine receptor list is in no way complete.

1.3.3.1 C-X-C chemokine receptors

By 1991, the effects of the C-X-C chemokines had been attributed to their ability to bind to and activate receptors on the surface of the neutrophils (Moser *et al.* 1991). It was Holmes *et al.* that first reported the cloning of a high affinity IL-8 receptor from a neutrophil cDNA library. This receptor bound IL-8 with high affinity but did not bind other members of the C-X-C chemokine group (Holmes *et al.* 1991).
Melanocyte growth stimulatory activity (MGSA), another C-X-C member, had also been observed to bind to human neutrophils and compete for the IL-8 binding sites and so this group went on to isolate and clone a second, distinct high affinity IL-8 receptor which also had a high affinity site for MGSA (Lee *et al.* 1992). These receptors were named IL-8 type A and IL-8 type B receptors respectively. They did not bind other, unrelated chemotactic factors such as C5a or fMLP.

Both of these receptors possess seven transmembrane domains which are typical of receptors linked to heterotrimeric G-proteins (Boulay *et al.* 1990) (Gerard & Gerard, 1994). The role of the G-proteins in IL-8 receptor signal transduction was further confirmed by the inhibition of the calcium responses by pertussis toxin, known to inactivate the G_i- and G_o-type G-proteins by adenosine diphosphate (ADP)-ribosylation (Thelen *et al.* 1988).

Table 1.2 - Members of the human chemokine family. The chemokine family is divided into three main sub-families, namely the C-X-C, C-C and C, based on the arrangement of the first two cysteine residues. The C-X-C group is then further sub-divided into E-L-R and non-E-L-R members, depending on the presence/absence of the E-L-R residues immediately upstream of the C-X-C motif. Three of the C-X-C (E-L-R) group are also separate as they are all derived from platelet basic protein (PBP) and are breakdown products of each other i.e. β-TG is a cleavage product of PBP which is cleaved to produce CTAP-III which is in turn cleaved to produce NAP-2. The asterisks indicate chemokines which have, to date, only been identified in mouse or rat. The chemokines in bold were discovered following the start of this project.

Chemokine sub-family	Chemokine name	Reference
C-X-C (E-L-R)	IL-8	(Baggiolini *et al.* 1989)
	MGSA/gro α, β, γ	(Thomas *et al.* 1991)
	ENA78	(Walz *et al.* 1991)
	SDF-1α/1β	(Tashiro *et al.* 1993)
	GCP-2	(Proost *et al.* 1993)
	MIP-2	(Wolpe *et al.* 1989)
	***LIX**	(Smith & Herschman, 1995)
C-X-C (E-L-R) (platelet basic protein cleavage products)	β-TG	(Begg *et al.* 1978)
	CTAP-III	(Castor *et al.* 1983)
	NAP-2	(Farber, 1993)
C-X-C (non E-L-R)	PF-4	(Deuel *et al.* 1977)
	IP-10	(Luster & Ravetch, 1987)
	Mig	(Farber, 1993)

Table 1.2 (cont'd.) - Members of the chemokine family.

Chemokine sub-family	Chemokine name	Reference
C-C	RANTES	(Schall *et al.* 1988)
	MCP-1	(Leonard & Yoshimura, 1990)
	MCP-2	(Van Damme *et al.* 1992)
	MCP-3	(Van Damme *et al.* 1992)
	MCP-4	(Uguccioni *et al.* 1996)
	MIP-1α	(Obaru *et al.* 1986)
	MIP-1β	(Lipes *et al.* 1988)
	Eotaxin	(Ponath *et al.* 1996)
	I309/TCA3	(Miller *et al.* 1989)
	***MRP-1/C10**	(Orlofsky *et al.* 1994)
	***MRP-2**	(Youn *et al.* 1995)
	***CCF18**	(Hara *et al.* 1995)
	***MIP-1γ**	(Mohamadzadeh *et al.* 1996)
C	**Lymphotactin**	(Kennedy *et al.* 1995)

Very recently (September 1996), two more C-X-C chemokine receptors were identified. These have been named CXC R3 and CXC R4 respectively. CXC R3 has been observed to bind monokine induced by gamma interferon (Mig) and IP-10. CXC R4 was originally described as an orphan chemokine receptor in 1993 by Loetscher *et al.* and called leukocyte-expressed seven-transmembrane-domain receptor (LESTR). Until recently, no ligands for this receptor had been found but it has now been observed to bind the C-X-C chemokine stromal-cell-derived factor-1 (SDF-1) (Loetscher *et al.* 1996) (Bleul *et al.* 1996). Due to the nomenclature adopted for these two receptors, the IL-8 RA and RB receptors are now also known as CXC R1 and CXC R2 respectively (Gordon Conference on Chemokines, 1996).

1.3.3.2 C-C chemokine receptors

The C-C chemokines were found to bind to high affinity receptors on the cell surface (Yoshimura & Leonard, 1990). The presence of multiple receptors for the various members of the C-C family were discovered by desensitisation experiments in which the calcium responses become desensitised upon repeated stimulation of the same receptor.

It was Neote *et al.* who cloned the first C-C chemokine receptor (CC CKR 1) from a number of different hematopoetic cell types known to respond to the C-C chemokines (Neote *et al.* 1993). This receptor was found to produce a calcium response to very low concentrations of MIP-1α and RANTES when transfected into human embryonic kidney (HEK) 293 cells. No effects were seen in the presence of either C-X-C chemokines or unrelated chemoattractant factors such as fMLP or C5a. However, this receptor did not account for the potent responses to other C-C chemokines such as MCP-1 (Van Riper *et al.* 1993).

In the last two years a number of C-C chemokine receptors have been identified. Firstly, Charo *et al.* cloned two high affinity MCP-1 receptors from monocytes. These receptors possess alternatively spliced C-terminal tails and have been named C-C CKR 2A and 2B (Charo *et al.* 1994). When expressed into human embryonic kidney (HEK) 293 cells they were confirmed as high affinity binding sites for MCP-1 and they were also found to bind MCP-3 (Franci *et al.* 1995).

Within the last year, two groups have identified a CC chemokine receptor (CC CKR 3) which is only found in eosinophils although there is some dispute as to the ligands which bind to it (Combadiere *et al.* 1995a) (Kitaura *et al.* 1996) (Daugherty *et al.* 1996) (Combadiere *et al.* 1995b). Two other CC chemokine receptors, namely CC CKR 4 and CC CKR 5, have also been identified. CC CKR 4 is found primarily on basophils. CC CKR 5 has not been fully characterised (Combadiere *et al.* 1995a) (Combadiere *et al.* 1995b) although, recently Samson *et al.* reported a new CC chemokine receptor which has been identified in a promyeloblastic cell line and which they have tentatively named CC CKR 5 (Samson *et al.* 1996). The CC CKR 5 has also recently been identified on human monocytes, macrophages and T-cells (Deng *et al.* 1996).

All the known C-C chemokine receptors possess the same structure as the C-X-C chemokine receptors with seven transmembrane spanning domains linked to heterotrimeric G-proteins. This was confirmed by the sensitivity of the C-C chemokine responses to pertussis toxin (Sozzani *et al.* 1991).

1.3.3.3 Other chemokine receptors

There is one other main chemokine receptor known as the Duffy antigen receptor for chemokines (DARC). This is the same as the Duffy blood group antigen that is found on red blood cells and has been found to be the binding site for both C-X-C and C-C chemokines as well as the malaria parasite *Plasmodium vivax* (Horuk *et al.* 1993).

Table 1.3 - Members of the chemokine receptor family. Where binding affinities have not been reported the chemokines which elicit a response have been included as binding to the receptors. The receptors in the shaded boxes were discovered following the start of this project.

Chemokine	Receptor	Agonist	Expression	Reference
C-X-C	IL-8 RA (CXC R1)	High affinity IL-8 Low affinity MGSA	Neutrophils, T cells	Holmes *et al.* 1991
	IL-8 RB (CXC R2)	High affinity IL-8 High affinity MGSA	Neutrophils, T cells	Lee *et al.* 1992
	CXC R3	Binds Mig and IP-10	?	Loetscher *et al.* 1996
	CXC R4	Binds SDF-1	T cells, monocytes	Bleul *et al.* 1996
C-C	CC CKR 1	High affinity MIP-1α, RANTES and MCP-3 Low affinity MCP-1 and MIP-1β	HL60, U937, THP-1, B cells, T cells , neutrophils, monocytes, (eosinophils?)	Neote *et al.* 1993 Gao *et al.* 1993 Combadiere *et al.* 1995
	CC CKR 2A and 2B	High affinity MCP-1 Binds MCP-3 with slightly lower affinity	Monocytes, THP-1, MonoMac 6	Charo *et al.* 1994 Franci *et al.* 1995
	CC CKR 3	Binds eotaxin High affinity for eotaxin, RANTES and MCP-3	Eosinophils, small amount on neutrophils and monocytes	Kitaura *et al.* 1996 Daugherty *et al.* 1996
	CC CKR 4	Binds MIP-1α, RANTES and MCP-1	KU812, T cells, B cells, monocytes, platelets	Power *et al.* 1995
	CC CKR 5	Binds MIP-1α, MIP-1β and RANTES	? KG-1A promyeloblastic cell line Monocytes, macrophages, T cells	Combadiere *et al.* 1995 Samson *et al.* 1996 Deng *et al.* 1996
Promiscuous	DARC	Binds all C-X-C and C-C chemokines except MIP-1α and MIP-1β	Red blood cells, postcapillary venule endothelial cells, T cells, B cells	Darbonne *et al.* 1991 Hadley *et al.* 1994 Wallace *et al.* 1995

Unlike all the other chemokine receptors DARC appears to have nine transmembrane spanning domains and is not coupled to heterotrimeric G-proteins (Chaudhuri *et al.* 1993). Recently, binding studies have been performed to examine which of the C-X-C and C-C chemokines bind to DARC (Chaudhuri *et al.* 1994). The only chemokines found not to bind to this promiscuous receptor were MIP-1α, MIP-1β and the C chemokine lymphotactin (Szabo *et al.* 1995).

Whether DARC tranduces a biological signal is not clear and the exact role of DARC has not yet been fully elucidated. It has been proposed as a 'sink' for IL-8 and other chemokines, keeping their levels in the circulation low (Darbonne *et al.* 1991). Interestingly, IL-8 which is bound to DARC on red blood cells cannot facilitate neutrophil activation. The fact that DARC is also expressed on endothelial cells lining the postcapillary venules indicates that it may play a role in the chemokine gradient and the leukocyte-endothelial cell interaction (Hadley *et al.* 1994). Also, the expression of DARC on T cells and the finding that antibodies to DARC induce proliferation of certain T cell subsets, could indicate a biological role for DARC (Wallace *et al.* 1995) (Horuk *et al.* 1996).

1.3.4 Role of chemokines in disease

The chemokines have been identified in a number of different diseases in the past few years. For example, the C-X-C chemokine IL-8 has been identified in psoriatic scales as well as in patients with eczema (Schroder *et al.* 1992) and in patients with rheumatoid arthritis (Seitz *et al.* 1991), osteoarthritis and gout (Rampart *et al.* 1992). IL-8 is also increased in alveolar macrophages from patients with idiopathic pulmonary fibrosis (Carre *et al.* 1991).

C-C chemokines have also been identified in a number of diseases including asthma (Sousa *et al.* 1994), rheumatoid arthritis (Koch *et al.* 1992) and atherosclerosis (Nelken *et al.* 1991). Interestingly, RANTES, MIP-1α and MIP-1β have been identified

as major human immunodeficiency virus (HIV)-suppressive factors (Cocchi *et al.* 1995) and in Vol 381 of Nature published in June 1996 Dragic *et al.* reported that the CC CKR5 had been identified as the second receptor for primary non-syncytium-inducing HIV-1 (NSI HIV-1) strains and MIP-1α, MIP-1β and RANTES all inhibited infection of the $CD4^+$ T cells at the virus entry stage (Dragic *et al.* 1996).

1.4 Role of adhesion molecules and chemokines in leukocyte migration

It is interesting to note that both chemokines and adhesion molecules play an important and closely related role in leukocyte migration. The chemokines are important in both the upregulation of integrins on the surface of leukocytes as well as in directing the leukocytes to the site of inflammation (see figure 1.2). The chemokine induced direction of leukocytes can occur by one of two mechanisms, chemotaxis or haptotaxis. In chemotaxis, the cells move in the direction of increasing concentration of a soluble chemokine. Its concentration is greatest at its site of production and diffuses away (Devreotes & Zigmond, 1988). In haptotaxis, the chemokine becomes bound to the surface of the endothelial cells, for example to surface proteoglycans. A chemokine gradient is still formed but it is fixed to the endothelial layer. There is still some controversy as to which mechanism is demonstrated *in vivo* (Rot, 1992). The haptotactic response may be more important when considering the fact that soluble chemokines released into the blood stream would be rapidly diluted and swept downstream. Although this would provide a gradient it may be quickly dispersed. Chemokines attached to the endothelium would be able to form a gradient but it would be more easily accessible to the leukocytes as they rolled along the endothelium.

Therefore, the adhesion and subsequent transmigration of leukocytes into a site of inflammation does not only require one signal but a number of consecutive signals which together lead to leukocyte migration.

1.5 Monocyte chemotactic peptide-1

Of particular interest in this study was the C-C chemokine MCP-1 and its effects on human monocytes.

1.5.1 Identification of MCP-1

In 1973, Altman *et al.* reported that the stimulation of blood mononuclear leukocytes by tuberculin caused the production of a chemotactic factor for human monocytes (Altman *et al.* 1973). They later referred to this molecule as leukocyte-derived chemotactic factor (LDCF) (Altman, 1978). It was ten years later that Yoshimura *et al.* purified monocyte chemotactic activity (MCA) from PHA stimulated human peripheral blood mononuclear cells (PBMC) (Yoshimura *et al.* 1989b). Due to the limited amount of MCA which could be collected from these cells this group went on to discover that the human gliomal cell line U-105MG also secreted this protein (Yoshimura *et al.* 1989a). Large enough quantities were then obtained to purify, sequence and clone this protein which they named MCP-1. This group also proposed that MCP-1 is the monocyte chemotactic activity first identified by Altman *et al.* some fifteen years earlier.

Concurrently, Valente *et al.* isolated a monocyte chemotactic peptide from baboon aortic smooth muscle cells which they called smooth muscle cell derived chemotactic factor (SMC-CF) (Valente *et al.* 1988). Examination of the peptide sequence identified that SMF-CF was identical to MCP-1.

1.5.2 MCP-1 structure

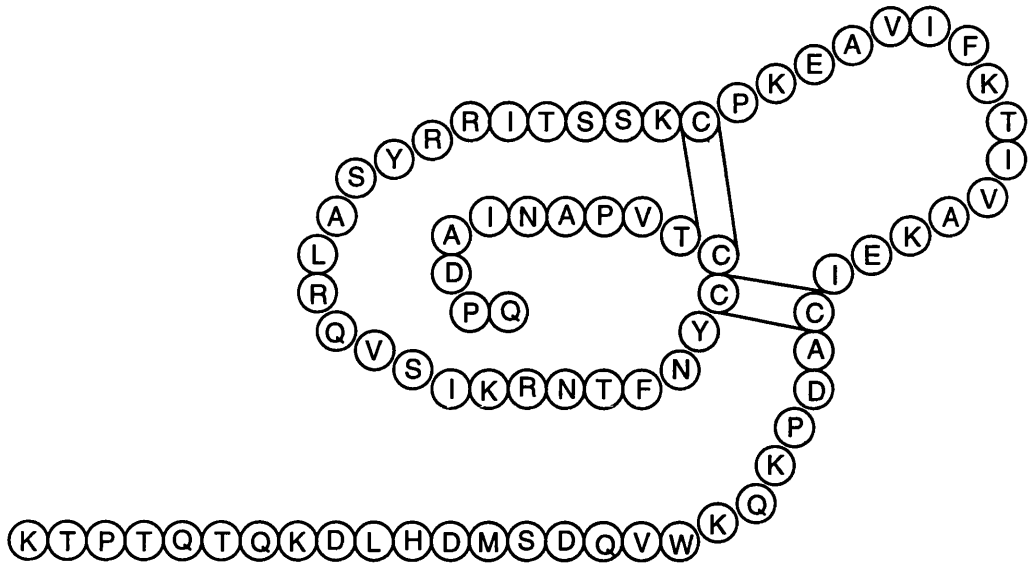


Figure 1.2 - Amino acid sequence and structure of MCP-1. The parallel lines indicate where the four cysteine residues form disulphide bonds. The one letter amino acid codes are as given in the abbreviations list. (Figure taken from Baggiolini *et al.* 1994).

Figure 1.2 shows the structure of MCP-1 which is characterised by two intrachain disulphide bonds. The precursor for MCP-1 is a polypeptide of 99 amino acids. The mature 76 amino acid peptide possesses a long C-terminal sequence and a short N-terminal sequence. Synthesis of MCP-1 analogues which lack the amino terminal region have identified it as crucial for receptor binding and for functional activity (Gong & Clark-Lewis, 1995) (Zhang *et al.* 1994). Deletion of residues 7-10 prevented receptor desensitisation and removal of residues 1-6 inhibited functional activity. Although these N-terminal residues are important for functional activity other regions of the protein are also required as a peptide of residues 1-10 was inactive by itself. These structure-activity relationships have also led to the proposal that MCP-1 forms dimers in physiological conditions (Zhang *et al.* 1994). By chemically cross-linking MCP-1 dimers and studying their activity compared to non-cross-linked MCP-1, the

role for MCP-1 dimerisation was investigated (Zhang & Rollins, 1995). Both the cross-linked and non-cross-linked MCP-1 peptides were equipotent at inducing monocyte chemotaxis, indicating that MCP-1 is functionally active in the dimeric form. More extensive studies using heteronuclear multidimensional nuclear magnetic resonance have confirmed that MCP-1 does form homodimers, although the exact role of monomers versus dimers *in vivo* is still not entirely clear (Handel & Domaille, 1996).

MCP-1 occurs as two electrophoretically distinct forms due to its glycosylation. Both the 9kDa and the 13kDa MCP-1 variants are equipotent biologically (Jiang *et al.* 1991).

1.5.3 Biological effects of MCP-1

MCP-1 is a particularly potent basophil degranulator and monocyte activator (Sozzani *et al.* 1991) (Alam *et al.* 1992). The main effects of MCP-1 on basophils include the rise in intracellular free calcium, histamine release and the generation of leukotriene C4 (LtC4) in interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte-macrophage-colony stimulating factor (GM-CSF) pre-treated basophils (Bischoff *et al.* 1992). The activation and subsequent release of pro-inflammatory agents from basophils is of primary importance in the maintenance of the inflammatory response.

The effects of MCP-1 on monocytes have not been studied in detail. When MCP-1 was first purified from PHA stimulated human blood mononuclear leukocytes it was observed to induce a chemotactic response in human monocytes (Yoshimura *et al.* 1989b). In 1990, Zachariae *et al.* discovered that MCP-1 activated monocyte functions such as cytostatic activity against tumour cells and lysosomal enzyme and superoxide anion release (Zachariae *et al.* 1990). In 1991, Sozzani *et al.* extended this study to characterise the MCP-1-induced monocyte chemotaxis and intracellular calcium rises and to identify their sensitivity to pertussis toxin (Sozzani *et al.* 1991).

This group went on to look at the rise in intracellular calcium and identified a calcium influx pathway activated by MCP-1, unrelated to voltage operated calcium channels (Sozzani *et al.* 1993).

At about the same time, the effects of MCP-1 on the cell surface expression of certain adhesion molecules involved in monocyte migration and diapedesis were investigated (Jiang *et al.* 1992). The results showed that MCP-1 was capable of upregulating two of the β2 integrins studied, further identifying a role for MCP-1 in inflammation via the activation of monocytes. However, the signal transduction pathways involved in this adhesion molecule upregulation and other monocyte functional responses were not investigated and, until the start of my project , this was the only data available on the activation of monocytes by MCP-1.

1.6 Signalling pathways

The binding of an agonist to its receptor expressed on the surface of a cell leads to the activation of intracellular signalling pathways followed by the induction of a functional response. The initial coupling of the receptor to the signalling cascade can occur via the activation of either protein tyrosine kinases (PTK) or through the activation of G-proteins.

PTKs can be subdivided into two groups, the transmembrane receptor family and the cytosolic non-receptor family (review (Hunter, 1995)). Signalling of the receptor PTK family involves ligand-mediated receptor dimerisation which results in the transphosphorylation of the receptor subunits within the dimer and activation of the PTK. Some examples of receptors with this intrinsic PTK activity include the epidermal growth factor receptor (EGFR), the platelet derived growth factor receptor (PDGFR) and the insulin receptor. The receptors coupled to the non-receptor PTKs do not possess a PTK catalytic domain but signal through the interaction of one of the receptor subunits with the non-receptor PTKs. By far the most well characterised family of non-receptor PTKs is the Src family of tyrosine kinases. Examples of the receptors which signal through non-receptor PTKs include cytokine receptors, such as IL-3 and IL-5, as well as the T cell and B cell antigen receptors. It is the Jak and the Src family non-receptor PTKs respectively that serve as the signalling molecules for these receptors.

There are a number of receptors which, rather than coupling to PTKs, couple to G-proteins (reviews (Birnbaumer, 1990) (Clapham & Neer, 1993)) . G-proteins consist of three subunits, the α, β and γ subunits. When a G-protein-coupled receptor, which usually possesses a seven transmembrane domain topology, is bound by an agonist, it leads to the activation of the G-protein. Upon activation, the α subunit binds GTP and dissociates from the $\beta\gamma$ subunit. It was originally proposed that only the α subunit

controlled the activity of the effectors, following the observation that the α subunit activated adenylyl cyclase and retinal cyclic GMP phosphodiesterase. However, it is now clear that both the α and the $\beta\gamma$ subunits can activate a number of cellular enzymes and ion channels. It is the intrinsic GTPase activity of the α subunit that leads to GTP hydrolysis, the reassociation of the α with the $\beta\gamma$ subunit and inactivation of the G-protein. Some examples of the receptors that are coupled to G-proteins include chemokine receptors, the fMLP receptor and the C5a receptor.

The following section will discuss two signalling pathways, namely the relatively well characterised phospholipase C (PLC) pathway and the putative signalling cascade regulated by phosphatidylinositol 3-kinase (PI 3-kinase). Both PTKs and G-proteins have been implicated in the activation of PLC and PI 3-kinase isoforms.

1.6.1 Phospholipase C

Elevations in intracellular calcium have been found to be instrumental in a number of different cellular processes including fertilisation, cell growth, smooth muscle contraction, neuronal conductance and sensory perception (Berridge, 1993). It has been during the last fifteen years that a role for PLC and its products have been identified (Berridge, 1984) (Streb *et al.* 1983). PLC catalyses the formation of inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG) from the hydrolysis of their membrane bound substrate phosphatidylinositol (4,5) bisphosphate (PtdIns (4,5)P_2). The IP_3 then binds to IP_3 receptors on the endoplasmic reticulum and stimulates the release of intracellular calcium stores and, in some cases, regulates calcium entry (see section 1.6.1.3). DAG binds to and activates several isoenzymes of the protein kinase C (PKC) superfamily (Nishizuka, 1988).

1.6.1.1 PLC subtypes and their activation

The PLC family is sub-divided into three groups (β, γ, δ) which differ in the method of their activation. Each of these groups consists of more than isoform. The members of the PLC family are summarised in table 1.4.

Table 1.4 - PLC isoforms identified in mammalian tissues. Summary of the PLC isoforms and their methods of activation. Activators in brackets activate these isoforms to a lesser extent

PLC isoforms	Activators
β_1	α-subunit of Gq G-protein (βγ-subunit of Gi-G-protein)
β_2	
β_3	α-subunit of Gq G-protein and βγ-subunit of Gi G-protein
γ_1	Receptor -coupled or non-receptor -coupled tyrosine kinase
γ_2	receptors via SH2 domain of PLC
δ_1	? - calcium -sensitive isoform of the enzyme?
δ_2	
δ_3	

1.6.1.1.1 PLC β activation

The PLC-β family is sub-divided into β_1, β_2 and β_3, as well as no receptor potential (norp) A, found in *Drosophila*. They are activated by heterotrimeric G proteins (review Sternwies & Smrcka, 1992). Activation of a G-protein-coupled receptor leads to the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit. This is facilitated by Mg^{2+} which binds to the G(GDP) complex and causes GDP to dissociate and allows GTP to bind to the α subunit (Birnbaumer, 1990). This leads to the dissociation of the α-subunit from the βγ-subunit. PLC β isoforms can be activated by both the α subunits of the Gq class and by the βγ subunits, depending on the agonist (Wu *et al.* 1993) (Katz *et al.* 1992). For example, the α subunit activates

PLC β following thromboxane A_2, bradykinin and histamine receptor stimulation (Shenker *et al.* 1991) (Rozengurt, 1986). The βγ subunits activate PLC β following the muscarinic m_2 and IL-8 receptor stimulation (Park *et al.* 1993) (Thelen *et al.* 1988). These subunits bind to different sites on the PLCβ molecule, with the βγ subunits binding to the N-terminal and the α subunit binding to the C-terminal region of PLC β (Clapham & Neer, 1993). Once activated, the PLC β can hydrolyse PtdIns(4,5)P_2 to produce IP_3 and DAG. This reaction is turned off by the hydrolysis of the GTP to GDP by the GTPase activity associated with the α-subunit. The α-subunit then recombines with the βγ-subunit and the complex is inactive once more (figure 1.3a).

1.6.1.1.2 PLC γ activation

The PLC-γ family is sub-divided into γ_1 and γ_2 and these PLC members are activated by tyrosine kinase-linked receptors. Specific tyrosine residues become phosphorylated by either receptor-coupled tyrosine kinases, as for platelet derived growth factor (PDGF), or by non-receptor-coupled tyrosine kinases such as *src* kinases in the case of the T cell receptor (TCR) (Samelson *et al.* 1990). These phosphorylated tyrosine residues provide docking sites for the *src* homology 2 (SH2) domains of the PLC-γ. PLCγ, which is usually present in the cytosol, then becomes located very close to the plasma membrane where its substrate is positioned. Once the enzyme has become attached to these docking sites the PLC-γ is itself phosphorylated on specific tyrosine residues and then begins to hydrolyse PtdIns(4,5)P_2. This reaction is switched off by the uncoupling of the PLC-γ from the tyrosine residues on the receptor and dephosphorylation (figure 1.3b).

1.6.1.1.3 PLC δ activation

The exact role of the PLC-δ is not well characterised. There are three members of this family, namely δ_1, δ_2 and δ_3. It is possible that it may be a calcium sensitive form

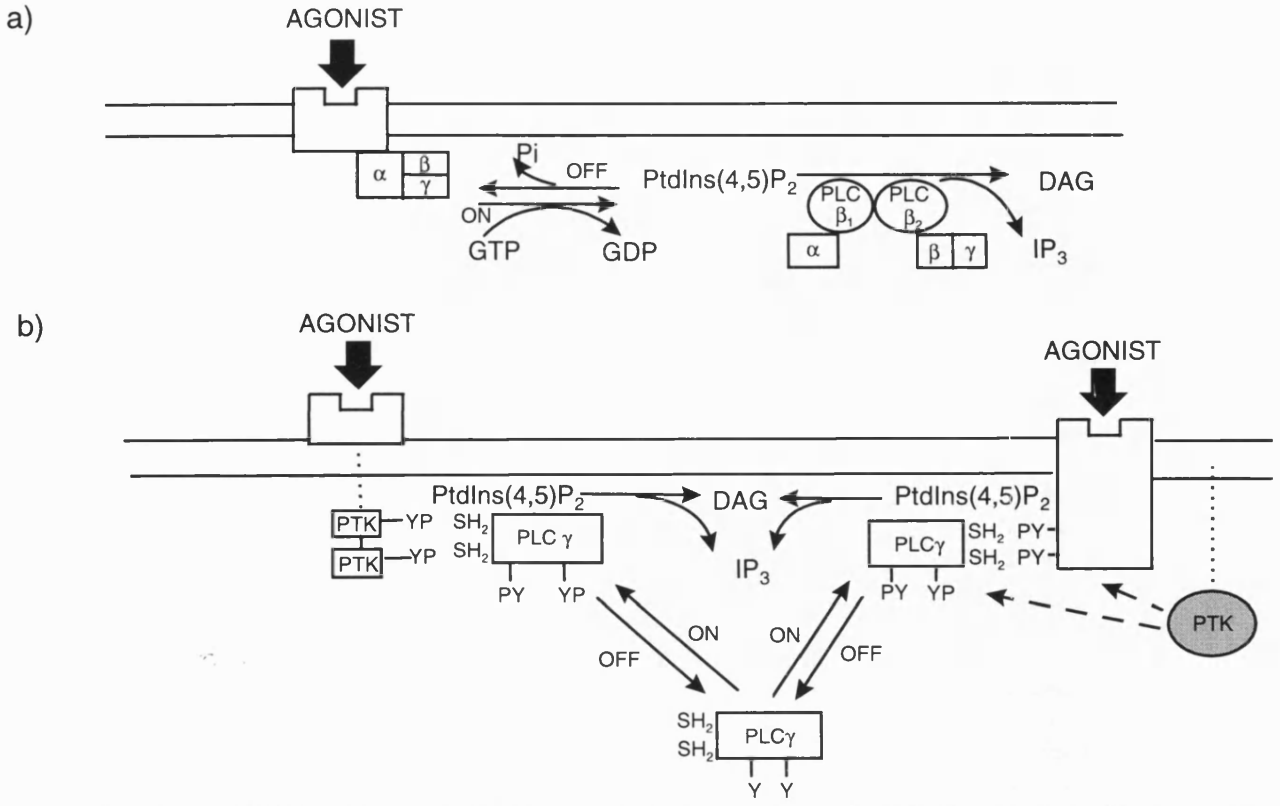


Figure 1.3 - Activation of PLC. a) The activation of PLC-β by heterotrimeric G-proteins. Upon receptor activation the Gα subunit dissociates from the Gβγ subunit and both of these subunits can then activate different isoforms of PLCβ depending on the cell type. The hydrolysis of GTP for GDP on the α subunit inactivates the system. b) The activation of PLC-γ by either receptor-coupled (clear box) or non-receptor-coupled (filled circle) tyrosine kinases. Specific residues on the receptor become phosphorylated which then provide a docking site for PLCγ. Upon phosphorylation of PLC PtdIns(4,5)P_2 is hydrolysed. The reaction is turned off by dephosphorylation and uncoupling of PLC (adapted from Berridge, 1993).

of the enzyme due to the specific motifs it contains and its enzymatic activity observed in the micromolar concentration range of calcium (Bairoch & Cox, 1990).

The PLC product IP_3 subsequently binds to receptors on the intracellular calcium stores, most probably the endoplasmic reticulum and induces calcium mobilisation (Berridge & Irvine, 1989). The structure of the IP_3 receptors and their role in calcium mobilisation has been well characterised.

1.6.1.2 IP_3 receptors and their function

The IP_3 receptor possesses two properties, namely an IP_3 binding property and a calcium release channel property. There are three main members of the IP_3 receptor family, IP_3 receptor (IP_3R) 1,2 and 3 and they are all characterised as large, tetrameric proteins with a molecular weight of about 300kDa and six membrane spanning regions (Furuichi *et al.* 1989). The C-terminal domain of each tetramer is the membrane spanning domain which anchors the receptor in the membrane and the N-terminal domain lies free in the cytoplasm and contains the IP_3 binding site (Mignery & Sudhof, 1990). Upon binding of IP_3 to its receptor, the receptor undergoes a conformational change which most probably leads to the opening of a channel in the centre of the IP_3 receptor. However, there is some controversy as to whether channel opening depends on the sequential binding of IP_3 to all four binding sites, each binding step inducing partial opening of the channel, or whether the binding of a single IP_3 molecule is sufficient for channel opening (Meyer & Stryer, 1990) (Watras *et al.* 1991).

Once IP_3 has bound to its receptor it can then either be metabolised by a 5-phosphatase to produce inositol (1,4) bisphosphate (IP_2) (Connolly *et al.* 1986) or it can be phosphorylated by a 3-kinase to produce inositol (1,3,4,5) tetrakisphosphate (IP_4) and even higher polyphosphoinositols (Koppler *et al.* 1993).

As well as calcium mobilisation from the intracellular calcium stores there is also a second source of intracellular calcium, namely influx from the extracellular medium. Upon activation of the cell, calcium has been demonstrated to enter the cells through channels operated by a number of different mechanisms such as voltage operated channels (VOC), receptor operated channels (ROC) and second messenger operated channels (SMOC).

There are six classes of VOCs, namely L, N, P, T, R and Q, which have been defined on the basis of their physiological and pharmacological properties (review (Godfraind & Govoni, 1995)). Interestingly, monocytes, as well as neutrophils, are two of a number of blood derived cells which do not possess VOCs (Merritt *et al.* 1989) (Sozzani *et al.* 1993) as defined by the lack of calcium influx in the presence of depolarising concentrations of potassium as well as by the lack of inhibition of agonist-induced elevations of $[Ca^{2+}]_i$ by agents such as nifedipine, diltiazem and verapamil, which interact with VOCs (Hagiwara & Byerly, 1981). ROCs have been proposed to be activated by a variety of agonists on cell types such as endothelial cells, platelets and parotid acinar cells as well as monocytes (Mendolesi *et al.* 1991) (Sozzani *et al.* 1993). With regard to the SMOCs, IP_3 and IP_4 have both been thoroughly investigated (Irvine, 1990). Although IP_3 and IP_4-sensitive calcium channels have been identified on the surface of a number of cells, such as lymphocytes (Khan *et al.* 1992), olfactory cells (Kalinoski *et al.* 1992) and endothelial cells (Luckhoff & Clapham, 1992) this is not the only method by which calcium influx can be influenced by IP_3. Depletion of the intracellular calcium stores has also been found to induce a influx of calcium and it was Putney in 1986 who adopted the term 'capacitative calcium entry' (Putney, Jr. 1986).

1.6.1.3 Capacitative calcium entry

When certain hormones, growth factors or other agonists induce a rise in intracellular calcium via the generation of IP_3, the response is biphasic. The initial increase is due the mobilisation of the intracellular calcium stores. This is followed by a second, prolonged phase which is the result of calcium entry (Westwick & Poll, 1986). In 1992, Hoth and Penner named the current induced by calcium entry through these channels the calcium-release-activated calcium current (I_{CRAC}) (Hoth & Penner, 1992). This entry of calcium is dependent upon initial store depletion, as demonstrated by the calcium ATPase inhibitor, thapsigargin, which can induce the same response by depleting the calcium stores but preventing refilling (Putney *et al.* 1981) (Clementi *et al.* 1995). This calcium entry can also be induced by IP_3, as intracellular application of IP_3 mimics the response and the IP_3 receptor blocker, heparin inhibits the calcium entry (Bird *et al.* 1992). However, in some cells neither IP_3 nor IP_4 were found to induce calcium entry (Murphy *et al.* 1995).

A large number of experiments have been performed over the last few years to try to establish the mechanism for this calcium entry. The various models which have been proposed to explain how information on the filling state of the calcium stores is transmitted to the membrane can be separated into two groups, those based on the presence of diffusible factors and those dependent on protein-protein interactions and conformational coupling (review (Berridge, 1995)).

There are two versions of the model based on the activation of capacitative calcium entry by diffusible factors. Firstly, the injection of the non-hydrolysable analogues of GTP or GDP, namely guanosine 5'-3-*O*-(thio)triphosphate (GTPγS) and guanosine 5'-*O*-(thio)diphosphate (GDPβS), were found to inhibit the calcium entry thus indicating a role for GTP hydrolysis (Bird & Putney, 1993). Other groups have postulated that these guanine analogues may cause disruption of a small GTP binding protein (SMG)

indicating a direct role for this SMG in calcium entry (Fasolato *et al.* 1993). However, these results must be interpreted cautiously, as these non-metabolisable guanine nucleotides may also interfere with other G-protein -coupled events such as the activation of PLC and the subsequent activation of PKC isotypes by DAG, resulting in the inhibition of capacitative calcium entry.

Secondly, a diffusible factor was identified by Randriamampita and Tsien (Randriamampita & Tsien, 1993). Following calcium depletion of a Jurkat lymphocyte cell line the cells were lysed and the crude extract applied to three different cell types. It induced a sustained elevation of intracellular calcium. This calcium influx factor (CIF) as it was called, was proposed to be a low molecular mass phosphorylated compound that is stored in the endoplasmic reticulum and is released upon store depletion. More recent work has identified the presence of at least two factors in this crude cell extract. One factor induces calcium influx when applied extracellularly and the second factor when applied intracellularly (Thomas & Hanley, 1995). It is the latter of the two activities which appears to be the one originally isolated by Randriamampita and Tsien, with a molecular weight of ≈ 500 (Randriamampita & Tsien, 1993). However, with the suggestion that Jurkat cells release acetylcholine which could be acting on the recipient cells (Murphy *et al.* 1995), the purification and identification of CIF is essential before this model can proceed.

The more direct role for IP_3 in the calcium entry has been proposed to be via a protein-protein interaction (review (Berridge, 1995)). Briefly, it is thought that the large head structure of the IP_3 receptor integrates a number of both positive and negative signals which it then transmits to the calcium channel via a conformational change. One of the main feedback signals is thought to be calcium itself (Zweifach & Lewis, 1995). For example, depletion of the intracellular calcium stores and low concentrations of calcium in the cellular cytosol lead to the transfer of this information

through the IP_3 receptor to the calcium channel. High concentrations of calcium in the cellular cytosol lead to a negative feedback effect preventing calcium influx.

However, there are a number of other components which may act to regulate this capacitative calcium entry. For example, protein phosphorylation has been reported to play an important role in capacitative calcium entry (Hoth & Penner, 1992). Both serine/threonine kinases, such as PKC, and tyrosine kinases have been demonstrated to regulate capacitative calcium entry, although the precise target of phosphorylation is not entirely clear (Petersen & Berridge, 1994) (Lee *et al.* 1993). The channel itself and the IP_3 receptor may both be likely phosphorylation targets. IP_3 binding to its receptor may also directly activate the CRAC channel. It has also been proposed that the presence of different isoforms of the IP_3 receptor may mean that they possess different functions. This led to experiments to identify whether the calcium release from the intracellular stores and the transfer of information to the channel were performed by different receptors. Results so far have indicated that the type 1 receptor may mediate calcium release and a separate receptor, perhaps type 3, may mediate calcium entry (Berridge, 1995). It is also possible that one IP_3 receptor is responsible for both calcium influx and calcium release from intracellular stores, but when it is coupled to the CRAC channel, its release properties are lost. The lack of calcium binding sites on the parts of the IP_3 receptor which face the lumen of the calcium store indicate the possibility that another calcium binding protein, for example calsequestrin or calreticulin, may sense the luminal calcium concentration and pass the message onto the IP_3 receptor. The recent identification of an IP_4 binding protein may implicate IP_4 in the transfer of information from the IP_3 receptor to the CRAC channel (Cullen *et al.* 1995). The exact nature of the protein-protein coupling between the IP_3 receptor and the CRAC channel is not known and far more investigation is required before a definitive answer can be given (see figure 1.4 for a summary of the calcium pathways).

The actual structure of the channel involved in this calcium entry has recently been carefully scrutinised. Work carried out on *Drosophila* photoreceptors led to the discovery of a transient receptor potential (*trp*) gene product which functions as a plasma membrane channel (Phillips *et al.* 1992). Concurrently, a second channel, named the *trp-like* (*trpl*) (Phillips *et al.* 1992) channel was identified. When *trp* and *trpl* were expressed into Sf9 cells both were demonstrated to be calcium channels. These two channels were observed to differ with respect to their cation selectivity and activation following depletion of the intracellular calcium stores. *trp* was found to be a selective channel for calcium and magnesium ions and to be sensitive to store depletion. In contrast, *trpl* was a non-selective ion channel which was not sensitive to store depletion and was activated by agonists of G-protein-coupled receptors via IP_3 (Vaca *et al.* 1994) (Hu & Schilling, 1995). The *trp* channel has been demonstrated to possess a higher conductance than the CRAC channel and to be less specific but it is possible that the calcium store-sensitive *trp* channel may be the functional analogue of the CRAC channel involved in capacitative calcium entry. Interestingly, a human homologue of the *trp* channel has recently been reported by two separate groups (Wes *et al.* 1995) (Zhu *et al.* 1995) and has been named transient receptor potential channel-related protein 1 (TRPC1) and human *trp* homologue 1 (Htrp1) respectively. It is proposed to be a member of a family of at least six related human proteins (Wes *et al.* 1995). Expression of the Htrp1 into COS-M6 cells demonstrated the ability to increase calcium influx into these cells by 75% (Zhu *et al.* 1996). Also, anti-*trp* sequences are capable of suppressing capacitative calcium entry in a fibroblast cell line, thus providing the first tentative links between the human *trp* homologues and capacitative calcium entry. It is important to note that both the Drosophila *trp* and *trpl* and the human homologue, trpc1/Htrp, contain three ankyrin repeats. The IP_3 receptors expressed in a number of mammalian tissues have been demonstrated to bind directly to ankyrin (Joseph & Samanta, 1993), thus providing a possible method

by which the IP_3 receptors may mediate activation of the channel, as proposed in the conformational change hypothesis.

1.6.1.4 Activation of PKC isoforms by DAG

The second product of PtdIns(4,5)P_2 hydrolysis by PLC is DAG, which can activate certain isotypes of PKC. Not only can DAG be produced as a result of PtdIns(4,5)P_2 hydrolysis, but it can also be produced as a result of hydrolysis of phosphatidylcholine (PtdC) by the PC-specific PLC. However, the time-course of the production of DAG from these two sources varies, with the production of DAG from inositol phospholipids being very rapid in onset and the DAG generated from PtdC relatively slow in onset.

To date, eleven isoforms of PKC have been identified and they differ quite markedly in their requirements for activation. Table 1.5 lists the PKC isoenzymes identified thus far and some of the various factors required for their activation (review (Nishizuka, 1995)). Briefly, the conventional PKCs (α, β, γ) require the presence of both calcium and DAG for activation. Activation of PKCs δ, ε, η, θ and μ is independent of calcium but they are still activated by DAG. The atypical PKCs (ζ and λ) are not activated by either calcium or DAG. Phorbol esters have been demonstrated to substitute for DAG in the activation of the DAG-sensitive isoforms of PKC. Interestingly, the product of PI 3-kinase, namely PtdIns(3,4,5)P_3, has also been demonstrated to activate the PKC δ, ε, η and ζ (see section 1.6.2.4.4).

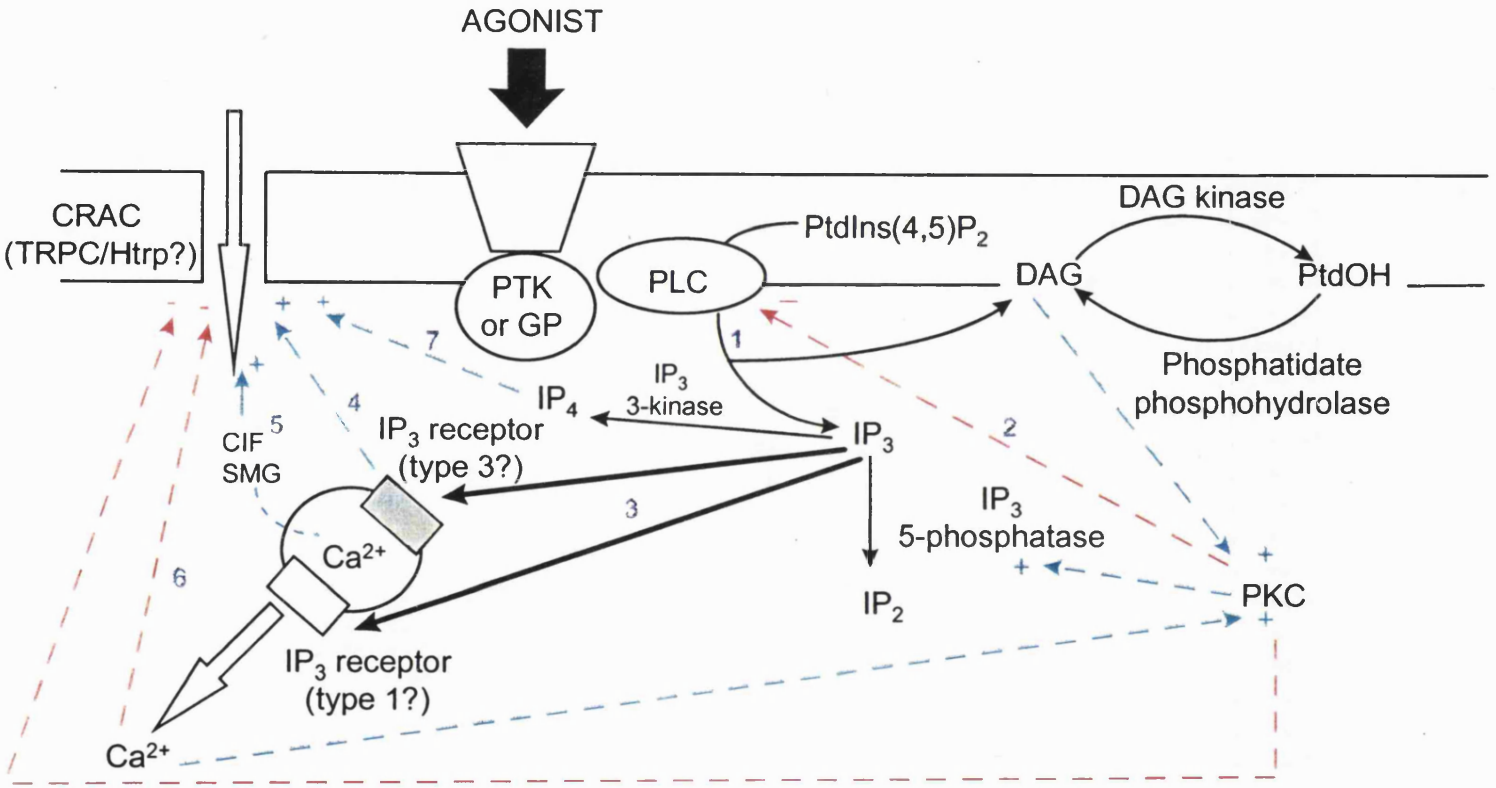


Figure 1.4 - Pathway to show the calcium mobilisation and calcium influx interrelationship. The stimulatory effects are shown in green and the inhibitory effects are shown in red. 1) IP_3 is generated by the hydrolysis of $PtdIns(4,5)P_2$ by PLC. 2) PKC, certain isoforms of which are activated by calcium as well as the other product of PLC, DAG, can inhibit calcium mobilisation by inhibiting PLC and increasing IP_3 breakdown. There have also been some reports that PKC can inhibit the CRAC channel. 3) IP_3 induces calcium mobilisation from intracellular stores by binding to its receptor. 4) An IP_3 receptor, either the same one that induces calcium mobilisation or a distinct IP_3 receptor, may activate the CRAC channel by conformational coupling. 5) Factors may be released by the stores upon calcium depletion to activate the CRAC channel. 6) High cytosolic concentrations of calcium may inhibit calcium entry via the CRAC channel. 7) IP_4 may play a role in the activation of the CRAC channel via an IP_4 binding protein.

Table 1.5 - PKC isoforms found in mammalian tissues. The PKC isoforms and some of their activators are given below (adapted from (Nishizuka, 1995)). These isoforms are also differentially activated by a variety of different lipids.

PKC isotypes	Activators
α βI βII γ	Calcium, DAG, phorbol esters
δ ε η	DAG, phorbol esters, PtdIns(3,4,5)P_3
θ	?
μ	?
ζ	PtdIns(3,4,5)P_3
λ	?

Calcium plays a very important role in a number of different cellular responses and investigations into the source of an agonist-induced calcium rise may provide vital evidence as to the activation pathway utilised by the agonist on a given cell type.

1.6.2 Phosphatidylinositol 3-kinase

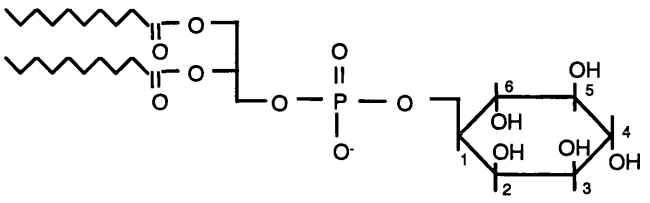
1.6.2.1 Formation of D-3 PtdIns lipids

PI 3-kinase is a lipid kinase which phosphorylates PtdIns lipids at the D-3 position of the inositol ring (figure 1.5) (reviews (Downes & Carter, 1991) (Stephens *et al.* 1993)). The potential products of this phosphorylation are phosphatidylinositol (3)-phosphate (PtdIns (3) P), phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol (3,4,5)-trisphosphate (PtdIns (3,4,5)P_3). Although these three products can all potentially originate from PI 3-kinase activation, the rates at which

these product levels change following agonist stimulation vary quite markedly. The levels of PtdIns (3)P remain unchanged following agonist stimulation, PtdIns(3,4,5)P_3 levels rise very rapidly and the increase in PtdIns(3,4)P_2 levels are often delayed (Stephens *et al.* 1991) (Kucera & Rittenhouse, 1990). The rapid and transient increases in PtdIns(3,4,5)P_3 implicate this molecule as a possible signalling molecule, as does the agonist induced increase in PtdIns(3,4)P_2. The fact that very small or non-existent changes in PtdIns(3)P have been detected in response to agonists indicates a lack of importance of this inositol lipid as a signalling molecule. The relative sizes of the phospholipid pools may explain the lack of detection of agonist-induced changes in PtdIns(3)P. The PtdIns(3)P pool is usually large and as only small changes in the other 3-phosphorylated lipids are required for cellular activation, then a significant difference in the levels of PtdIns(3)P may not be detected. However, it is interesting to note that a mammalian PtdIns-specific PI 3-kinase and yeast PI 3-kinase activity (Vps34) have been identified, both of which can only phosphorylate in the D-3 position of PtdIns (sections 1.6.2.2.4 and 1.6.2.2.5) (Auger *et al.* 1989).

The possibility that the agonist-induced changes in these D-3 phosphorylated lipids may be due to other enzymes have been studied in detail by a number of groups, using [^{32}P]P_i or [γ-^{32}P]ATP labelled cells (Stephens *et al.* 1991) (Hawkins *et al.* 1992) (Stephens *et al.* 1993c). Experiments performed under pre-isotopic equilibrium conditions demonstrated that the generation of PtdIns(3,4,5)P_3 was by the 3-phosphorylation of PtdIns(4,5)P_2, consistent with the presence of a PI 3-kinase. However, the origin of PtdIns(3,4)P_2 and PtdIns(3)P was not clear, particularly in light of the evidence for the presence of a PtdIns(3,4,5)P_3 5-phosphatase (Jackson *et al.* 1995) (Woscholski *et al.* 1995) (Ono *et al.* 1996) and a PtdIns(3,4)P_2 4-phosphatase (Stephens *et al.* 1991). Despite the ambiguity over the possible sources of PtdIns(3)P and PtdIns(3,4)P_2, there is little doubt that the presence of a PI 3-kinase is responsible for the agonist-induced increases in PtdIns(3,4,5)P_3.

a)



PtdIns

b)

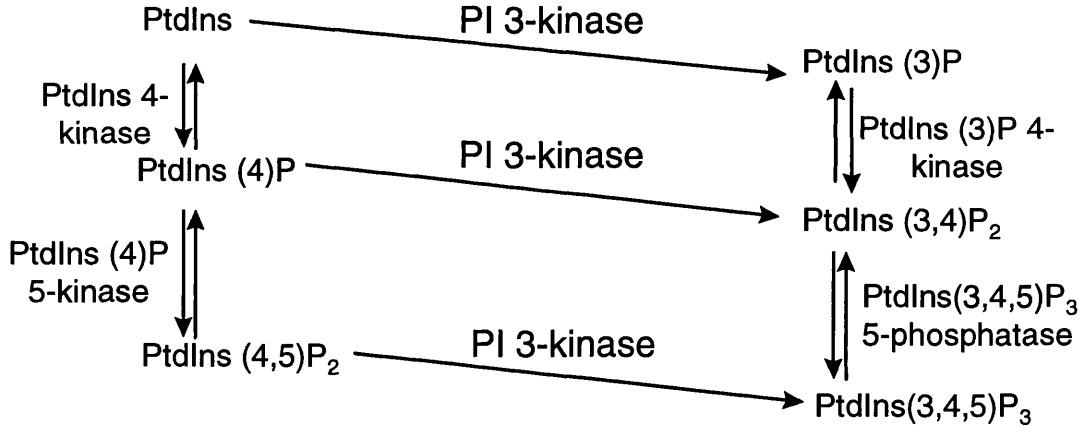


Figure 1.5 - The synthesis of D-3 phosphorylated phosphoinositides.
a) The structure of PtdInsindicating the numbering on the inositol ring.
b) The products formed by the phosphorylation in the D-3 position by PI 3-kinase. The other enzymes which also contribute to the formation and breakdown of these products are also shown.

1.6.2.2 The PI 3-kinase family

1.6.2.2.1 PI 3-kinase identification

PI 3-kinase was first isolated from cells tightly attached to two virally encoded PTKs, v-*src* and v-*ros* (Sugimoto *et al.* 1984). The relationship between this PI 3-kinase and PTKs was further clarified by the fact that the PDGF receptor, which possess intrinsic PTK activity, could recruit PI 3-kinase within less than a minute after PDGF stimulation (Whitman *et al.* 1987) (Kaplan *et al.* 1987). Further experiments were then performed using a mutated PDGF receptor. Mutation of the PDGF receptor in the 'kinase insert' domain, which has been demonstrated to be the region which interacts with PI 3-kinase, prevented PDGF-induced cell proliferation (Coughlin *et al.* 1989).

About the same time, a uniquely polar phospholipid was identified in human neutrophils which increased rapidly following fMLP stimulation and, from the chromatographic properties of its head group, it was identified as PtdIns(3,4,5)P_3 (Traynor-Kaplan *et al.* 1988). The identification of the agonist-induced generation of a second 3-phosphorylated lipid, namely PtdIns (3,4)P_2 in the same cells (Traynor-Kaplan *et al.* 1989), led to the connection between the PI 3-kinase activity and the generation of these lipids. This connection was further corroborated by the demonstration that PDGF could not only stimulate the translocation of PI 3-kinase to its receptor but also the rapid appearance of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 (see table 1.6 for a complete listing of the PI 3-kinase family members identified to date).

1.6.2.2.2 PTK/SH2 -coupled PI 3-kinase structure

The PI 3-kinase activity first identified as being tightly associated with PTKs, i.e. the PDGF receptor-associated PI 3-kinase, has been fairly well characterised and will be referred to as the PTK/SH2-coupled PI 3-kinase throughout this thesis. The PTK/SH2-coupled PI 3-kinase has been purified from rodent liver (Carpenter *et al.*

1990), mouse fibroblasts (Escobedo *et al.* 1991) and bovine brain (Otsu *et al.* 1991). It consists of two subunits, p85 and p110.

The p85 subunit is the regulatory subunit and two isoforms have been isolated from bovine brain, namely p85α and p85β (Otsu *et al.* 1991). They show extensive amino acid homology and both bind to the activated PDGF protein tyrosine kinase receptor. These p85 subunits contain several domains of homology to other proteins, namely one SH3 domain and two SH2 domains (src homology domains 3 and 2), which are involved in the regulation of PI 3-kinase (section 1.6.2.3.1), in addition to a breakpoint cluster region (BCR)-like domain. The latter has been found to confer GTPase activity for small molecular weight GTP-binding proteins (Fry *et al.* 1992).

Upon isolation of the p85 subunit, a second protein with a molecular weight of 110kDa was observed to be tightly associated with this p85 subunit (Carpenter *et al.* 1990). Following the expression of this 110kDa protein into Sf9 cells, p110 was demonstrated to possess PI 3-kinase activity and designated the catalytic subunit of PI 3-kinase (Hiles *et al.* 1992). A second p110 isoform has more recently been identified, namely p110β from a human library. This also has PI 3-kinase activity and associates with the p85 subunit (Hu *et al.* 1993). The p85 and p110 subunits associate with each other via one region corresponding to the sequences between the two SH2 domains of p85 (Hu *et al.* 1993) (Klippel *et al.* 1994).

It is interesting to note that the p85 subunit of PI 3-kinase, as well as facilitating the interaction of the p110 subunit with activated receptors, has also been proposed as a mediator of other protein-protein interactions (Kapeller & Cantley, 1994). For example, p85 has been reported to function as an adaptor molecule, linking the activated insulin receptor to the multifunctional SH2 and SH3 domain binding protein p62, as well as to the GTPase-activating protein (GAP) (Sung *et al.* 1994).

The p110 subunit also has the potential for cross-talk with other pathways present within cells. For example, the GTP binding protein Ras has been demonstrated to bind directly with the p110 subunit of PI 3-kinase in a GTP-dependent manner. The dominant negative Ras mutant has been demonstrated to inhibit the accumulation of D-3 phospholipids in PC12 cells following growth factor stimulation (Rodriguez-Viciana *et al.* 1994). The proteins encoded by the *ras* genes serve as essential transducers of diverse physiological signals (Lowy & Willumsen, 1993) and the observation that the dominant negative Ras mutant can inhibit PKB activation indirectly points to the possibility that the interaction of p110 with Ras may contribute to the activation of PKB (Downward, 1995).

Other than the lipid kinase activity of the p110 subunit of PI 3-kinase, it has also been demonstrated to possess a serine kinase activity, similar to the Vps15 which interacts with Vps34 in yeast (section 1.6.2.2.5). This intrinsic protein kinase activity has been proposed to play a role in the regulation of PI 3-kinase *in vivo* (section 1.6.2.3.1).

1.6.2.2.3 G-protein -coupled PI 3-kinase

A significant number of receptors which belong to the seven transmembrane G-protein-coupled receptor family can also stimulate PI 3-kinase. Examples of these are fMLP (Stephens *et al.* 1991), LtB_4 (Traynor-Kaplan *et al.* 1989), adenosine triphosphate (ATP), platelet activating factor (PAF) (Stephens *et al.* 1993a), C5a (Dobos *et al.* 1992) and thrombin (Kucera & Rittenhouse, 1990). None of these receptors possess intrinsic PTK activity nor do they associate with non-receptor PTKs. Therefore, the heterotrimeric G-proteins with which they interact must lead to PI 3-kinase activation. This theory was further substantiated by two other findings. Firstly, mastoparan, a peptide which activates heterotrimeric G-proteins, can induce rapid activation of PI 3-kinase in human neutrophils (Norgauer *et al.* 1992). Secondly,

fMLP-, PAF-, and ATP-stimulated PI 3-kinase activity in neutrophils and thrombin-stimulated PI 3-kinase activity in platelets were all completely abrogated or reduced respectively following pertussis toxin pre-treatment (Traynor-Kaplan *et al.* 1988) (Stephens *et al.* 1993b) (Stephens *et al.* 1993a) (King *et al.* 1991).

It was originally thought that this G-protein-coupled PI 3-kinase also possessed a heterodimeric structure consisting of a p85 and a p110 subunit. However, more recent evidence has lead to the conclusion that this PI 3-kinase only possesses a p110 subunit which is separate from that seen in the PTK/SH2-coupled PI 3-kinase (Stoyanov *et al.* 1995). This p110 subunit was called p110γ. Throughout this thesis, this PI 3-kinase isoform will be referred to as PI 3-kinase γ.

1.6.2.2.4 PtdIns-specific PI 3-kinase

A PtdIns-specific PI 3-kinase has recently been identified in mammalian cells (Stephens *et al.* 1994) (Volina *et al.* 1995). This isoform of PI 3-kinase has a molecular weight of 100kDa and does not appear to associate with the p85 regulatory subunit. *In vivo*, this PtdIns-specific PI 3-kinase is detected in a complex with a 150kDa protein. The PtdIns-specific PI 3-kinase demonstrates a similar sensitivity to wortmannin as is observed with the PTK/SH2-coupled PI 3-kinase but, unlike the PTK/SH2-coupled PI 3-kinase, the PtdIns-specific PI 3-kinase was relatively insensitive to non-ionic detergents.

1.6.2.2.5 PI 3-kinase activity in yeast

A PI 3-kinase activity was identified in yeast following the observation that a large number of *Saccharomyces cerevisiae* mutants are specifically defective in vacuolar protein sorting (Bankaitis *et al.* 1986) (Rothman *et al.* 1986). Analyses of two such *Vps* genes, namely Vps34 and Vps15, demonstrated that these genes encode homologues of a PI 3-kinase and a serine/threonine kinase respectively (Herman &

Emr, 1990) (Hiles *et al.* 1992). Vps34 is homologous to the p110 catalytic subunit of mammalian PTK/SH2-coupled PI 3-kinase, although, unlike the mammalian PTK/SH2-coupled PI 3-kinase, Vps34 can only phosphorylate in the D-3 position of PtdIns. Point mutations in the conserved region of the lipid kinase domain resulted in almost complete abrogation of the PI 3-kinase activity as well as severe defects in vacuolar protein sorting (Schu *et al.* 1993). Vps34 and Vps15 have been demonstrated to form a complex that is associated with the cytoplasmic face of the intracellular membrane fraction, most likely to be the Golgi apparatus (Herman *et al.* 1991). Not only does Vps15 recruit Vps34 to the membrane, it also serves to activate the PI 3-kinase activity in Vps34. The protein kinase activity of Vps15 has been demonstrated to be a prerequisite for association with and activation of Vps34 (Stack *et al.* 1995).

1.6.2.2.6 Other PI 3-kinase activities

A number of other putative PI 3-kinase activities have been identified, including Cpk in *Drosophila*, Cpk-m and p170 in mice as well as TOR1/TOR2, mTOR, RAFT, FRAP, ATM and DNA-PK_{CS}, all of which show homology with the p110 subunit of the PTK/SH2-coupled PI 3-kinase (Virbasius *et al.* 1996) (Molz *et al.* 1996) (Hartley *et al.* 1995) (Sabatini *et al.* 1995). However, no real functional evidence has yet been provided for these isoforms.

1.6.2.3 PI 3-kinase coupling and activation

1.6.2.3.1 PTK/SH2 -coupled PI 3-kinase

PI 3-kinase is predominantly a soluble enzyme which uses membrane-associated substrates. Therefore, the enzyme must translocate to and become associated with the membrane before it can phosphorylate the substrates. For the comparatively well characterised PTK/SH2-coupled PI 3-kinase, this appears to occur via tyrosine phosphorylated residues associated with the receptor. These proteins can either be

phosphorylated by a receptor-coupled PTK or a non-receptor-coupled PTK, such as *src*-type PTKs, in a similar manner to that seen by PLC γ (Cantley *et al.* 1991). The possible mechanism of activation of PI 3-kinase by these two systems is relatively similar and both are summarised in figure 1.6a.

The tyrosine phosphorylated proteins either on the receptor itself or on a target protein are thought to bind, via specific amino acid sequences, to the SH2 domains of the p85 subunit of PI 3-kinase. The sequences which bind the SH2 domains of PI 3-kinase have been studied in some detail and, to date, a number of binding motifs have been identified. For example, the binding of the PDGF receptor or the CD28, CTLA-4 and CD7 molecules on T cells to PI 3-kinase, appears to occur through the YXXM motif in the middle of the kinase domain of the PDGF receptor or in the cytoplasmic tails of the T cell molecules (Escobedo *et al.* 1991) (Kapeller & Cantley, 1994). This YXXM consensus sequence has also been observed to be present on PKC δ and PKC ε (Ettinger *et al.* 1996). The association of the hepatocyte growth factor receptor and the erythropoietin receptor have been demonstrated to be mediated by sequences such as YVXV and YLVL respectively (Ponzetto *et al.* 1993) (He *et al.* 1993) and TCR signal transduction has been proposed to be mediated by two YXXL/I repeats separated by 6-8 amino acid residues (Weiss & Littman, 1994). There is strong evidence that doubly phosphorylated peptides are more effective than singly phosphorylated peptides at activating PI 3-kinase (Carpenter *et al.* 1993a). It is thought that binding of the phosphopeptides to the SH2 domains of p85 induces a conformational change which allows activation of the p110 catalytic subunit.

The only major difference between the receptor-coupled PTK systems e.g. the PDGF and EGF receptors (reviewed (Cantley *et al.* 1991) and the non-receptor-coupled PTK systems e.g. fyn and lck in T cells (reviewed (Ward *et al.* 1996)) is the proteins which become phosphorylated.

Table 1.6 - PI 3-kinase family members. All the members of the PI 3-kinase family to date are given below along with the species in which they have been identified and the substrates upon which they can act. The shaded columns indicate the putative PI 3-kinase activities (adapted from Ward *et al.* 1996).

PI 3-kinase	Species	Substrate	Potential products
PTK/SH2 - coupled	Human	PtdIns, PtdIns(4)P, PtdIns(4,5)P_2	PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,4,5)P_3
PI 3-kinase γ	Human	PtdIns, PtdIns(4)P, PtdIns(4,5)P_2	PtdIns(3)P, PtdIns(3,4)P_2, PtdIns(3,4,5)P_3
PtdIns-specific	Human	PtdIns	PtdIns(3)P
Vps34	Yeast	PtdIns	PtdIns(3)P
Cpk	Drosophila	PtdIns, PtdIns(4)P	PtdIns(3)P, PtdIns(3,4)P_2
p170	Mouse	PtdIns, PtdIns(4)P	PtdIns(3)P, PtdIns(3,4)P_2
TOR1/TOR2	Yeast	Not known	Not known
mTOR	Human	Not known	Not known
RAFT	Rat	Not known	Not known (N.B. RAFT may co-precipitate with a PtdIns 4-kinase)
FRAP	Human	Not known	Not known
ATM	Human	Not known	Not known
DNA-PK_{CS}	Human	None	None

In the former system, it is the PTK or, in the case of the insulin receptor the insulin receptor substrate-1 (IRS-1) (Backer *et al.* 1992), which becomes phosphorylated and thus activates PI 3-kinase. With the non-receptor-coupled PTK system, either the PTK, the receptor or a target protein becomes phosphorylated which then subsequently activates PI 3-kinase via the SH2 domains.

The p85 subunit of PI 3-kinase possess one SH3 domain and one or more SH3 binding domains which may regulate PI 3-kinase activity. The function of SH3 domains in proteins is still not entirely clear but they are thought to function as potential sites for specific and tight interactions with certain proline-rich regions present in other proteins (Ren *et al.* 1993). The SH3 domain of p85, expressed as a GST fusion protein, has been demonstrated to bind the microtubule-associated protein, dynamin (Gout *et al.* 1993). Although the exact role for this interaction is not entirely clear, it may have important biological significance as the binding of dynamin to the SH3 domain further augments the GTPase activity of dynamin. The potential SH3 binding domains, or proline-rich regions, of p85 have also been demonstrated to interact with the SH3 domains in various non-receptor-coupled PTKs such as lck, fyn and lyn (Kapeller *et al.* 1994) (Pleiman *et al.* 1993) as well as the interaction of the SH3 domain of p85 with its own SH3 binding domains (Prasad *et al.* 1993). These interactions do not alter the activity of PI 3-kinase.

Another method of activation of the PTK/SH2-coupled PI 3-kinase may come from phosphorylation of the p85 subunit itself. p85 has been observed to be phosphorylated in response to the activation of many tyrosine kinases (Courtneidge & Heber, 1987) (Panayotou *et al.* 1992). The manganese-dependent serine-threonine kinase which is tightly associated with PI 3-kinase may also be important in cellular responses as it has been demonstrated to downregulate PI 3-kinase (Carpenter *et al.* 1993).

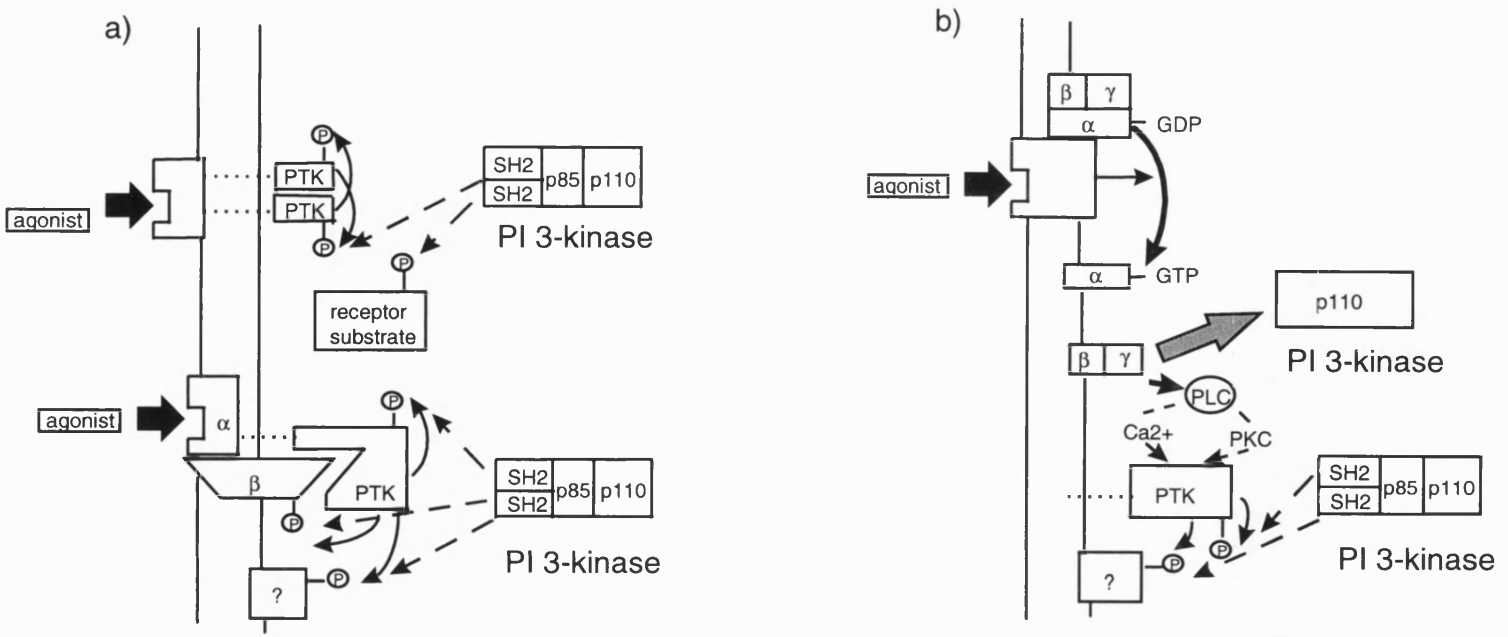


Figure 1.6 - The activation of PI 3-kinase isoforms. a) The activation of PI 3-kinase either by a receptor-coupled PTK or a non-receptor-coupled PTK. The phosphorylation of either the receptor itself or a target protein by either a receptor coupled PTK or a non-receptor coupled PTK allows the interaction of the phosphotyrosine with the SH2 domains of PI 3-kinase and thus PI 3-kinase activation. b) The activation of PI 3-kinase γ by G-protein-coupled receptors. The βγ subunits of the G-protein bind to and activate the PI 3-kinase γ. It has been proposed that the PTK/SH2 coupled PI 3-kinase can also be activated by G-protein coupled receptors via PKC and calcium activation of a PTK (adapted from Stephens *et al.* (1993)).

p85 has also been observed to be tyrosine phosphorylated in PDGF-stimulated, polyoma middle t antigen-transformed and nerve growth factor (NGF) stimulated cells as well as insulin stimulated cells (Cohen *et al.* 1990) (Courtneidge & Heber, 1987) (Kaplan *et al.* 1987) (Hayashi *et al.* 1993). The exact role of the phosphorylation of the p85 subunit is not yet clear, although it has been suggested to play a role in the dissociation of tyrosine phosphorylated residues as well as to regulate the interaction with catalytic subunits (Cohen *et al.* 1990) (Kavanaugh *et al.* 1992).

1.6.2.3.2 PI 3-kinase γ

Activation of PI 3-kinase γ is not completely understood. A schematic representation of its activation is given in figure 1.6b. Recent evidence has indicated that it is the βγ subunits which lead to the activation of PI 3-kinase. This has been demonstrated by the activation of PI 3-kinase in U937 cells, human platelets and neutrophils by purified bovine brain G

cells overexpressing src, the PKC phosphorylation site is a requirement for agonist-induced increases in cAMP (Gould *et al.* 1985) (Moyers *et al.* 1993).

1.6.2.4 Inhibitors of PI 3-kinase

The exact role of the D-3 phosphorylated products of PI 3-kinase is not entirely clear. They are definitely not substrates for PLC and thus do not play a role in inositol phosphate metabolism and calcium mobilisation (Lips *et al.* 1989) (Serunian *et al.* 1989). The similarities with the yeast Vps34, involved in vacuolar protein sorting, indicate that vesicular trafficking may be a role for mammalian PI 3-kinase (Hiles *et al.* 1992). However, further studies into the roles of PI 3-kinase and its products have been made possible by the discovery of the inhibitor wortmannin (Arcaro & Wymann, 1993) (review (Ui *et al.* 1995)).

1.6.2.4.1 Wortmannin

Wortmannin is a fungal metabolite which was originally described by Baggiolini *et al.* in 1987 as an inhibitor of respiratory burst in neutrophils (Baggiolini *et al.* 1987). It was subsequently found to inhibit other pathways including PLC and D (Bonser *et al.* 1993), myosin light chain kinase (Nakanishi *et al.* 1992) and pleckstrin phosphorylation in platelets (Yatomi *et al.* 1992), although the concentrations of wortmannin required for these effects were in the micromolar range. However, at 100nM or less, wortmannin has been found to be a potent inhibitor of PI 3-kinase by covalently interacting with the p110 catalytic subunit (Yano *et al.* 1993). Recently, a study carried out by Wymann *et al.* identified the residues required for the covalent binding of wortmannin to PI 3-kinase (Wymann *et al.* 1996). Wortmannin was found to bind to Lys-802 on the p110 α subunit of PI 3-kinase. Point mutation of this residue completely abolished wortmannin binding. However, the different isoforms of PI 3-kinase demonstrate differing sensitivities to wortmannin. The PTK/SH2-coupled PI 3-kinase is very sensitive to wortmannin with an IC_{50} of 10nM, as is the PtdIns-specific

PI 3-kinase with an IC_{50} of 2.5nM (Wolscholski *et al.* 1994) (Volinia *et al.* 1995). In contrast, PI 3-kinase γ is much less sensitive to wortmannin with an IC_{50} of 43nM on the purified enzyme (Stephens *et al.* 1994). Other PI 3-kinase isoforms such as Vps34, RAFT and FRAP are almost insensitive to wortmannin (Sabatini *et al.* 1995).

However, there are limitations as to the use of wortmannin as an indicator of PI 3-kinase activation due to its effective inhibition of at least two other signalling pathways, namely PtdIns 4-kinase and PLA_2 (Nakanishi *et al.* 1995) (Cross *et al.* 1995). The inhibition of these pathways may have profound effects on the PI 3-kinase pathway by affecting the generation of the PI 3-kinase substrate, PtdIns(4,5)P_2, or by affecting other pathways which may be directly or indirectly connected to PI 3-kinase.

1.6.2.4.2 Other inhibitors of PI 3-kinase

A novel PI 3-kinase inhibitor, namely 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was synthesised and demonstrated to inhibit purified bovine brain PI 3-kinase with an IC_{50} of 1.4μM (Vlahos *et al.* 1994). LY294002 was also found to inhibit the PI 3-kinase-dependent proliferation of cultured rabbit aortic smooth muscle cells as well as completely abolish fMLP-induced PI 3-kinase activity in human neutrophils. The effects of this inhibitor were also investigated on several other enzymes including PtdIns 4-kinase, src, MAP-kinase, S6 kinase, DAG kinase, PKA and PKC (Vlahos *et al.* 1994). None of these enzymes were significantly inhibited by LY294002.

There is another inhibitor of PI 3-kinase that has recently been identified. Demethoxyviridin is structurally related to wortmannin but is a distinct compound and was observed to inhibit purified PTK/SH2-coupled PI 3-kinase with an IC_{50} of 3.4nM (Wolscholski *et al.* 1994). However, demethoxyviridin, like wortmannin, has also been observed to inhibit PtdIns 4-kinase with an IC_{50} of approximately 50nM.

These inhibitors, particularly wortmannin, have been used to identify a number of functional responses which are dependent on PI 3-kinase activation (see table 1.7). These responses include fMLP activation of neutrophils, IL-2 production following CD28 ligation by B7 in T cells (Ward *et al.* 1995) and PDGF-mediated actin rearrangements in human fibroblasts and membrane ruffling in endothelial cells (Wymann & Arcaro, 1994) (Wennstrom *et al.* 1994).

1.6.2.4.3 Alternative approaches to studying PI 3-kinase

More recently, other approaches to the identification of PI 3-kinase-dependent responses have been initiated. A p85 deletion mutant, which lacks a small domain between the SH2 domains (inter-SH2 region) required for the interaction of the p85 subunit with the p110 catalytic subunit has been developed (Dhand *et al.* 1994). This prevents the interaction of the two subunits of PI 3-kinase and, when expressed in excess of the endogenous PI 3-kinase, can act as a dominant negative mutant due to its retained ability to associate with the phosphotyrosine binding sites that would activate endogenous PI 3-kinase. This dominant negative mutant has been observed to inhibit insulin-stimulated PtdIns(3,4,5)P_3 accumulation in U937 cells (Stephens, 1995).

A consitutively active PI 3-kinase has also been developed (Hu *et al.* 1995). In this mutant PI 3-kinase the catalytic p110 subunit is a chimeric protein in which the inter-SH2 region of the p85 subunit is covalently linked to its binding site on the p110 subunit. This consitutively active PI 3-kinase has been demonstrated to activate a number of signalling pathways, such as p70 S6 kinase, mitogen-activated protein (MAP) kinase and Ras, independent of growth factor stimulation (Weng *et al.* 1995).

1.6.2.4.4 Downstream effectors of PI 3-kinase

The development of PI 3-kinase inhibitors and active and dominant negative mutants has lead to a number of possible downstream effectors of PI 3-kinase being identified.

p70 S6 kinase is a serine/threonine kinase which phosphorylates the ribosomal protein S6. This results in enhanced mRNA translation and increased protein synthesis (Kozma and Thomas 1994) (review (Woodgett, 1994)). The activation of this kinase has not been well characterised but it is thought to involve multiple phosphorylation sites by more than one protein kinase and experiments using wortmannin have identified PI 3-kinase as an important regulator of p70 S6 kinase phosphorylation and activation (Parry & Ward, 1996) (Chung *et al.* 1994) (Monfar *et al.* 1995) (Kilgour *et al.* 1996). Also, mutations of the PDGF receptor, which prevent the binding of the p85 subunit of PI 3-kinase, prevented the activation of p70 S6 kinase by PDGF, further implicating PI 3-kinase in the activation of p70 S6 kinase (Chung *et al.* 1994).

Some groups have demonstrated that the macrolide antibiotic rapamycin also inhibits p70 S6 kinase activation (Downward, 1994). The proposed pathway involves the putative PI 3-kinase target of rapamycin (TOR). Rapamycin, when complexed with its binding protein FK506 binding protein (FKBP) 12, can bind to and inhibit the functions of TOR. Thus, the inhibition of both TOR and p70 S6 kinase by rapamycin implicates TOR in the activation of p70 S6 kinase. Similar results have also been observed with another putative PI 3-kinase in T cells (Brown *et al.* 1994). FK506-binding protein rapamycin-associated protein (FRAP) has been demonstrated to be required for p70 S6 kinase regulation and is sensitive to rapamycin (Brown *et al.* 1995). However, more work is required before a definitive answer can be given.

Another serine/threonine protein kinase has also been linked to PI 3-kinase activation. PKB is a product of the *Akt* gene and is ubiquitously expressed (Bellacosa *et al.* 1993). The catalytic domain of PKB is homologous to the catalytic domains of all members of the PKC family (Coffer & Woodgett, 1991). Wortmannin has been demonstrated to inhibit the PDGF-induced activation of PKB (Franke *et al.* 1995). The coexpression of a dominant negative mutant of PI 3-kinase has also been demonstrated to inhibit PDGF-induced PKB activation (Burgering *et al.* 1995). The link between the activation of PI 3-kinase and PKB by PDGF was also suggested by the fact that the addition of PtdIns(3)P induced activation of PKB in *in vitro* assays (Franke *et al.* 1995).

It is very interesting to note that Burgering *et al.* observed that the expression of a consitutively active PKB form resulted in the increased activity of p70 S6 kinase (Burgering *et al.* 1995). Therefore, the observations that both PKB and p70 S6 kinase are downstream effectors of PI 3-kinase and that PKB can activate p70 S6 kinase suggests that PKB may be positioned upstream of p70 S6 kinase.

Certain PKC isoforms have also been proposed as downstream effectors of PI 3-kinase following the observations that PI 3-kinase can associate with PKC δ in both GM-CSF stimulated TF-1 cells and PAF stimulated platelets (Ettinger *et al.* 1996) and that PtdIns(3,4,5)P_3 can stimulate PKC ε, δ, ζ and η isoforms in *in vitro* assays (Toker *et al.* 1994) (Nakanishi *et al.* 1993).

Finally, the identification of a proposed PtdIns(3,4,5)P_3 binding protein, namely centaurin-α, in rat brain has possibly identified another downstream effector for PtdIns(3,4,5)P_3 (Hammonds-Odie *et al.* 1996). Hammonds-Odie *et al.* proposed that

centaurin-α may have a role in mediating the downstream events activated following growth factor stimulation of PI 3-kinase in the brain.

Although the exact roles for PI 3-kinase and its products have not yet been fully identified, it is apparent that they may play an important role in the activation of a number of different cell types.

Table 1.7 - Responses observed to be potently inhibited by wortmannin. Some of the functional responses sensitive to nM concentrations of wortmannin are given below (adapted from Ward *et al.* 1996).

Functional response	Agonist	Wortmannin concentration (nM)	Reference
Respiratory burst from neutrophils	fMLP	5	Arcaro & Wymann, 1993
T cell chemotaxis	RANTES	5	Turner *et al.*, 1996
IL-2 production from T cells	CD28	10	Ward *et al.*, 1995
T cell proliferation	CD28 + CD3	10-100	Karnitz *et al.*, 1995
Actin rearrangements in fibroblasts	PDGF	5	Wymann & Arcaro, 1994
Membrane ruffling in endothelial cells	PDGF	10-100	Wennstrom *et al.*, 1994
β1 integrin upregulation in HL60 cells transfected with CD2/ $CD2^+$ T cells	Antibody stimulation of CD2	10-100	Shimizu *et al.*, 1995
Histamine secretion and leukotriene release from RBL cells	FcεR1	3	Yano *et al.*, 1993
Phagocytosis in U937 cells	Fcγ receptor induced	10-100	Ninomiya *et al.*, 1994

1.7 Monocyte functional changes

The main objective of this study was to characterise the action of the C-C chemokine MCP-1 on human monocytes and monocytic cell lines. Blood monocytes are immature cells that are produced in the bone marrow. They migrate into tissues and body cavities where they mature into macrophages. The monocyte-macrophage system plays a vital role in multiple host-defence mechanisms (Boggs & Winklestein, 1985). For example, macrophages can initiate the immune response by acting as antigen presenting cells (Scala & Oppenheim, 1985). Macrophages engulf antigens and partly degrade them. A fraction then reappears on the surface of the macrophage along with gene products of the major histocompatibility complex (MHC) which are then presented to the lymphocyte inducing an immune response. This immune response is further mediated by macrophages which produce IL-1. IL-1 production stimulates T cells to produce IL-2, thus promoting T cell proliferation. Monocytes are also potent effectors of the inflammatory processes by their capacity to release arachidonic acid-derived mediators and respiratory burst products. They are chiefly involved in defending those organisms which give rise to a chronic inflammation. Upon the development of an inflammatory response the monocytes will adhere to the endothelial layer, via the interaction of β-integrins with the Ig superfamily members expressed on the endothelial layer (see section 1.2) and move down the specific chemokine concentration gradient towards the site of inflammation. In the last stage, the monocyte will transmigrate through the endothelial layer into the tissue. The release of cytokines and arachidonic acid-derived mediators provide signals to the other cells involved in the inflammatory response and act as a positive feedback mechanism. The production of respiratory burst products such as superoxide ions is of great importance in the killing of bacteria. This is made very clear in patients suffering from the rare genetic disease, chronic granulomatous disease (CGD). Both the monocytes and the neutrophils lack one or more of the protein components of the

superoxide-generating system and the patients suffer repeated infections (Cross & Jones, 1991) (Hamers *et al.* 1984).

During the production of superoxide anions, there is an increase in oxygen uptake followed by the passing of two electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH), formed by the hexose monophosphate pathway, down two redox active components, namely a flavin adenine dinucleotide (FAD)-containing protein, and cytochrome b-245. This results in the eventual reduction of oxygen to produce superoxide (Cross & Jones, 1991). This system is known as NADPH oxidase. It becomes activated upon stimulation of the monocytes and the superoxide produced is released onto the outer face of the cell (Jones *et al.* 1993). The presence of superoxide dismutase (SOD) in the cells converts the superoxide ions into hydrogen peroxide (Root & Metcalfe, 1977).

Apart from the adhesion molecules that are upregulated on the surface of monocytes following stimulation there is another cell surface molecule which has been found to be upregulated on the surface of monocytes, namely CD23 (Vercelli *et al.* 1988). CD23 was first identified as a B cell activation marker and is a low affinity receptor for immunoglobulin E (IgE). It is also known as FCεRII (review (Delespesse *et al.* 1991)). CD23 is an integral type II membrane glycoprotein and several cytokines, including interleukin-4 (IL-4) (Vercelli *et al.* 1988), IL-3 (Alderson *et al.* 1992), GM-CSF and interferon-α (IFN-α) (Williams *et al.* 1992) stimulate its upregulation on monocytes. There are two isoforms of CD23, namely CD23a and b, that differ in their cytoplasmic domain and they have been shown to perform distinct functions (Yokota *et al.* 1988). It is the type b CD23 which is expressed on monocytes.

When CD23 expression is increased on the cell surface it can be cleaved into biologically active soluble fragments and this cleavage process has been

demonstrated to be involved in the differentiation of the myelomonocytic leukaemia cell line U937 into mature monocyte/macrophage cells (Ouaaz *et al.* 1993). CD23 upregulation has also been identified in phagocytosis of IgE-coated particles by monocytes (Boltz-Nitulescu *et al.* 1988).

The upregulation of this cell surface CD23 molecule appears to be important in the monocytes regulation in IgE -dependent diseases and its upregulation on monocytes from patients suffering from allergic asthma has already been confirmed (Demoly *et al.* 1994).

Due to the difficulty in obtaining large numbers of blood-derived monocytes, the monocytic cell line THP-1 was utilised in this study. THP-1 cells were originally derived from a patient suffering from acute monocytic leukaemia (Tsuchiya *et al.* 1980). They are a pre-monocytic cell line but, like monocytes, they possess Fc and C3b receptors. However, they lack surface and cytoplasmic immunoglobulins. They are a phagocytic cell line and can be differentiated into macrophage-like cells by factors such as phorbol ester (Tsuchiya *et al.* 1982).

1.8 Aims of study

1. To determine the binding of MCP-1 to the human monocytic cell line THP-1 and to human embryonic kidney (HEK) 293 cells stably transfected with the CC CKR 2 A or B, using both radioligand binding studies and biotinylated MCP-1 and FACS analysis.

2. To examine the increase in intracellular calcium in response to MCP-1 using fura-2 labelled cells and to explore the source of this calcium rise with the aid of various calcium influx and calcium mobilisation inhibitors in THP-1 cells compared to the transfected cells and human blood derived monocytes and to identify the possible role for PLC by studying IP_3 generation.

3. To demonstrate the activation of PI 3-kinase by MCP-1 in THP-1 cells compared with the transfected cells by monitoring the production of PI 3-kinase products and to identify whether the PTK/SH2 -coupled PI 3-kinase and/or the G-protein -coupled PI 3-kinase are activated following MCP-1 stimulation using both a whole cell assay and an *in vitro* lipid kinase assay.

4. To determine whether MCP-1 induces tyrosine phosphorylation of proteins in THP-1 cells and the MCP-1 receptor transfected cells and to identify the role of these proteins in PI 3-kinase activation using Western blotting techniques.

5. To study the activation of various functional responses in monocytes following MCP-1 stimulation including chemotaxis, adhesion molecule upregulation, superoxide release and low affinity IgE receptor expression and to try to identify a possible role for the signal transduction pathways activated by MCP-1.

cells overexpressing src, the PKC phosphorylation site is a requirement for agonist-induced increases in cAMP (Gould *et al.* 1985) (Moyers *et al.* 1993).

1.6.2.4 Inhibitors of PI 3-kinase

The exact role of the D-3 phosphorylated products of PI 3-kinase is not entirely clear. They are definitely not substrates for PLC and thus do not play a role in inositol phosphate metabolism and calcium mobilisation (Lips *et al.* 1989) (Serunian *et al.* 1989). The similarities with the yeast Vps34, involved in vacuolar protein sorting, indicate that vesicular trafficking may be a role for mammalian PI 3-kinase (Hiles *et al.* 1992). However, further studies into the roles of PI 3-kinase and its products have been made possible by the discovery of the inhibitor wortmannin (Arcaro & Wymann, 1993) (review (Ui *et al.* 1995)).

1.6.2.4.1 Wortmannin

Wortmannin is a fungal metabolite which was originally described by Baggiolini *et al.* in 1987 as an inhibitor of respiratory burst in neutrophils (Baggiolini *et al.* 1987). It was subsequently found to inhibit other pathways including PLC and D (Bonser *et al.* 1993), myosin light chain kinase (Nakanishi *et al.* 1992) and pleckstrin phosphorylation in platelets (Yatomi *et al.* 1992), although the concentrations of wortmannin required for these effects were in the micromolar range. However, at 100nM or less, wortmannin has been found to be a potent inhibitor of PI 3-kinase by covalently interacting with the p110 catalytic subunit (Yano *et al.* 1993). Recently, a study carried out by Wymann *et al.* identified the residues required for the covalent binding of wortmannin to PI 3-kinase (Wymann *et al.* 1996). Wortmannin was found to bind to Lys-802 on the p110 α subunit of PI 3-kinase. Point mutation of this residue completely abolished wortmannin binding. However, the different isoforms of PI 3-kinase demonstrate differing sensitivities to wortmannin. The PTK/SH2-coupled PI 3-kinase is very sensitive to wortmannin with an IC_{50} of 10nM, as is the PtdIns-specific

PI 3-kinase with an IC_{50} of 2.5nM (Wolscholski *et al.* 1994) (Volinia *et al.* 1995). In contrast, PI 3-kinase γ is much less sensitive to wortmannin with an IC_{50} of 43nM on the purified enzyme (Stephens *et al.* 1994). Other PI 3-kinase isoforms such as Vps34, RAFT and FRAP are almost insensitive to wortmannin (Sabatini *et al.* 1995).

However, there are limitations as to the use of wortmannin as an indicator of PI 3-kinase activation due to its effective inhibition of at least two other signalling pathways, namely PtdIns 4-kinase and PLA_2 (Nakanishi *et al.* 1995) (Cross *et al.* 1995). The inhibition of these pathways may have profound effects on the PI 3-kinase pathway by affecting the generation of the PI 3-kinase substrate, PtdIns(4,5)P_2, or by affecting other pathways which may be directly or indirectly connected to PI 3-kinase.

1.6.2.4.2 Other inhibitors of PI 3-kinase

A novel PI 3-kinase inhibitor, namely 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was synthesised and demonstrated to inhibit purified bovine brain PI 3-kinase with an IC_{50} of 1.4μM (Vlahos *et al.* 1994). LY294002 was also found to inhibit the PI 3-kinase-dependent proliferation of cultured rabbit aortic smooth muscle cells as well as completely abolish fMLP-induced PI 3-kinase activity in human neutrophils. The effects of this inhibitor were also investigated on several other enzymes including PtdIns 4-kinase, src, MAP-kinase, S6 kinase, DAG kinase, PKA and PKC (Vlahos *et al.* 1994). None of these enzymes were significantly inhibited by LY294002.

There is another inhibitor of PI 3-kinase that has recently been identified. Demethoxyviridin is structurally related to wortmannin but is a distinct compound and was observed to inhibit purified PTK/SH2-coupled PI 3-kinase with an IC_{50} of 3.4nM (Wolscholski *et al.* 1994). However, demethoxyviridin, like wortmannin, has also been observed to inhibit PtdIns 4-kinase with an IC_{50} of approximately 50nM.

These inhibitors, particularly wortmannin, have been used to identify a number of functional responses which are dependent on PI 3-kinase activation (see table 1.7). These responses include fMLP activation of neutrophils, IL-2 production following CD28 ligation by B7 in T cells (Ward *et al.* 1995) and PDGF-mediated actin rearrangements in human fibroblasts and membrane ruffling in endothelial cells (Wymann & Arcaro, 1994) (Wennstrom *et al.* 1994).

1.6.2.4.3 Alternative approaches to studying PI 3-kinase

More recently, other approaches to the identification of PI 3-kinase-dependent responses have been initiated. A p85 deletion mutant, which lacks a small domain between the SH2 domains (inter-SH2 region) required for the interaction of the p85 subunit with the p110 catalytic subunit has been developed (Dhand *et al.* 1994). This prevents the interaction of the two subunits of PI 3-kinase and, when expressed in excess of the endogenous PI 3-kinase, can act as a dominant negative mutant due to its retained ability to associate with the phosphotyrosine binding sites that would activate endogenous PI 3-kinase. This dominant negative mutant has been observed to inhibit insulin-stimulated PtdIns(3,4,5)P_3 accumulation in U937 cells (Stephens, 1995).

A consitutively active PI 3-kinase has also been developed (Hu *et al.* 1995). In this mutant PI 3-kinase the catalytic p110 subunit is a chimeric protein in which the inter-SH2 region of the p85 subunit is covalently linked to its binding site on the p110 subunit. This consitutively active PI 3-kinase has been demonstrated to activate a number of signalling pathways, such as p70 S6 kinase, mitogen-activated protein (MAP) kinase and Ras, independent of growth factor stimulation (Weng *et al.* 1995).

1.6.2.4.4 Downstream effectors of PI 3-kinase

The development of PI 3-kinase inhibitors and active and dominant negative mutants has lead to a number of possible downstream effectors of PI 3-kinase being identified.

p70 S6 kinase is a serine/threonine kinase which phosphorylates the ribosomal protein S6. This results in enhanced mRNA translation and increased protein synthesis (Kozma and Thomas 1994) (review (Woodgett, 1994)). The activation of this kinase has not been well characterised but it is thought to involve multiple phosphorylation sites by more than one protein kinase and experiments using wortmannin have identified PI 3-kinase as an important regulator of p70 S6 kinase phosphorylation and activation (Parry & Ward, 1996) (Chung *et al.* 1994) (Monfar *et al.* 1995) (Kilgour *et al.* 1996). Also, mutations of the PDGF receptor, which prevent the binding of the p85 subunit of PI 3-kinase, prevented the activation of p70 S6 kinase by PDGF, further implicating PI 3-kinase in the activation of p70 S6 kinase (Chung *et al.* 1994).

Some groups have demonstrated that the macrolide antibiotic rapamycin also inhibits p70 S6 kinase activation (Downward, 1994). The proposed pathway involves the putative PI 3-kinase target of rapamycin (TOR). Rapamycin, when complexed with its binding protein FK506 binding protein (FKBP) 12, can bind to and inhibit the functions of TOR. Thus, the inhibition of both TOR and p70 S6 kinase by rapamycin implicates TOR in the activation of p70 S6 kinase. Similar results have also been observed with another putative PI 3-kinase in T cells (Brown *et al.* 1994). FK506-binding protein rapamycin-associated protein (FRAP) has been demonstrated to be required for p70 S6 kinase regulation and is sensitive to rapamycin (Brown *et al.* 1995). However, more work is required before a definitive answer can be given.

Another serine/threonine protein kinase has also been linked to PI 3-kinase activation. PKB is a product of the *Akt* gene and is ubiquitously expressed (Bellacosa *et al.* 1993). The catalytic domain of PKB is homologous to the catalytic domains of all members of the PKC family (Coffer & Woodgett, 1991). Wortmannin has been demonstrated to inhibit the PDGF-induced activation of PKB (Franke *et al.* 1995). The coexpression of a dominant negative mutant of PI 3-kinase has also been demonstrated to inhibit PDGF-induced PKB activation (Burgering *et al.* 1995). The link between the activation of PI 3-kinase and PKB by PDGF was also suggested by the fact that the addition of PtdIns(3)P induced activation of PKB in *in vitro* assays (Franke *et al.* 1995).

It is very interesting to note that Burgering *et al.* observed that the expression of a consitutively active PKB form resulted in the increased activity of p70 S6 kinase (Burgering *et al.* 1995). Therefore, the observations that both PKB and p70 S6 kinase are downstream effectors of PI 3-kinase and that PKB can activate p70 S6 kinase suggests that PKB may be positioned upstream of p70 S6 kinase.

Certain PKC isoforms have also been proposed as downstream effectors of PI 3-kinase following the observations that PI 3-kinase can associate with PKC δ in both GM-CSFstimulated TF-1 cells and PAF stimulated platelets (Ettinger *et al.* 1996) and that PtdIns(3,4,5)P_3 can stimulate PKC ε, δ, ζ and η isoforms in *in vitro* assays (Toker *et al.* 1994) (Nakanishi *et al.* 1993).

Finally, the identification of a proposed PtdIns(3,4,5)P_3 binding protein, namely centaurin-α, in rat brain has possibly identified another downstream effector for PtdIns(3,4,5)P_3 (Hammonds-Odie *et al.* 1996). Hammonds-Odie *et al.* proposed that

centaurin-α may have a role in mediating the downstream events activated following growth factor stimulation of PI 3-kinase in the brain.

Although the exact roles for PI 3-kinase and its products have not yet been fully identified, it is apparent that they may play an important role in the activation of a number of different cell types.

Table 1.7 - Responses observed to be potently inhibited by wortmannin. Some of the functional responses sensitive to nM concentrations of wortmannin are given below (adapted from Ward *et al.* 1996).

Functional response	Agonist	Wortmannin concentration (nM)	Reference
Respiratory burst from neutrophils	fMLP	5	Arcaro & Wymann, 1993
T cell chemotaxis	RANTES	5	Turner *et al.*, 1996
IL-2 production from T cells	CD28	10	Ward *et al.*, 1995
T cell proliferation	CD28 + CD3	10-100	Karnitz *et al.*, 1995
Actin rearrangements in fibroblasts	PDGF	5	Wymann & Arcaro, 1994
Membrane ruffling in endothelial cells	PDGF	10-100	Wennstrom *et al.*, 1994
β1 integrin upregulation in HL60 cells transfected with CD2/ $CD2^+$ T cells	Antibody stimulation of CD2	10-100	Shimizu *et al.*, 1995
Histamine secretion and leukotriene release from RBL cells	FcεR1	3	Yano *et al.*, 1993
Phagocytosis in U937 cells	Fcγ receptor induced	10-100	Ninomiya *et al.*, 1994

1.7 Monocyte functional changes

The main objective of this study was to characterise the action of the C-C chemokine MCP-1 on human monocytes and monocytic cell lines. Blood monocytes are immature cells that are produced in the bone marrow. They migrate into tissues and body cavities where they mature into macrophages. The monocyte-macrophage system plays a vital role in multiple host-defence mechanisms (Boggs & Winklestein, 1985). For example, macrophages can initiate the immune response by acting as antigen presenting cells (Scala & Oppenheim, 1985). Macrophages engulf antigens and partly degrade them. A fraction then reappears on the surface of the macrophage along with gene products of the major histocompatibility complex (MHC) which are then presented to the lymphocyte inducing an immune response. This immune response is further mediated by macrophages which produce IL-1. IL-1 production stimulates T cells to produce IL-2, thus promoting T cell proliferation. Monocytes are also potent effectors of the inflammatory processes by their capacity to release arachidonic acid-derived mediators and respiratory burst products. They are chiefly involved in defending those organisms which give rise to a chronic inflammation. Upon the development of an inflammatory response the monocytes will adhere to the endothelial layer, via the interaction of β-integrins with the Ig superfamily members expressed on the endothelial layer (see section 1.2) and move down the specific chemokine concentration gradient towards the site of inflammation. In the last stage, the monocyte will transmigrate through the endothelial layer into the tissue. The release of cytokines and arachidonic acid-derived mediators provide signals to the other cells involved in the inflammatory response and act as a positive feedback mechanism. The production of respiratory burst products such as superoxide ions is of great importance in the killing of bacteria. This is made very clear in patients suffering from the rare genetic disease, chronic granulomatous disease (CGD). Both the monocytes and the neutrophils lack one or more of the protein components of the

superoxide-generating system and the patients suffer repeated infections (Cross & Jones, 1991) (Hamers *et al.* 1984).

During the production of superoxide anions, there is an increase in oxygen uptake followed by the passing of two electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH), formed by the hexose monophosphate pathway, down two redox active components, namely a flavin adenine dinucleotide (FAD)-containing protein, and cytochrome b-245. This results in the eventual reduction of oxygen to produce superoxide (Cross & Jones, 1991). This system is known as NADPH oxidase. It becomes activated upon stimulation of the monocytes and the superoxide produced is released onto the outer face of the cell (Jones *et al.* 1993). The presence of superoxide dismutase (SOD) in the cells converts the superoxide ions into hydrogen peroxide (Root & Metcalfe, 1977).

Apart from the adhesion molecules that are upregulated on the surface of monocytes following stimulation there is another cell surface molecule which has been found to be upregulated on the surface of monocytes, namely CD23 (Vercelli *et al.* 1988). CD23 was first identified as a B cell activation marker and is a low affinity receptor for immunoglobulin E (IgE). It is also known as FCεRII (review (Delespesse *et al.* 1991)). CD23 is an integral type II membrane glycoprotein and several cytokines, including interleukin-4 (IL-4) (Vercelli *et al.* 1988), IL-3 (Alderson *et al.* 1992), GM-CSF and interferon-α (IFN-α) (Williams *et al.* 1992) stimulate its upregulation on monocytes. There are two isoforms of CD23, namely CD23a and b, that differ in their cytoplasmic domain and they have been shown to perform distinct functions (Yokota *et al.* 1988). It is the type b CD23 which is expressed on monocytes.

When CD23 expression is increased on the cell surface it can be cleaved into biologically active soluble fragments and this cleavage process has been

demonstrated to be involved in the differentiation of the myelomonocytic leukaemia cell line U937 into mature monocyte/macrophage cells (Ouaaz *et al.* 1993). CD23 upregulation has also been identified in phagocytosis of IgE-coated particles by monocytes (Boltz-Nitulescu *et al.* 1988).

The upregulation of this cell surface CD23 molecule appears to be important in the monocytes regulation in IgE -dependent diseases and its upregulation on monocytes from patients suffering from allergic asthma has already been confirmed (Demoly *et al.* 1994).

Due to the difficulty in obtaining large numbers of blood-derived monocytes, the monocytic cell line THP-1 was utilised in this study. THP-1 cells were originally derived from a patient suffering from acute monocytic leukaemia (Tsuchiya *et al.* 1980). They are a pre-monocytic cell line but, like monocytes, they possess Fc and C3b receptors. However, they lack surface and cytoplasmic immunoglobulins. They are a phagocytic cell line and can be differentiated into macrophage-like cells by factors such as phorbol ester (Tsuchiya *et al.* 1982).

1.8 Aims of study

1. To determine the binding of MCP-1 to the human monocytic cell line THP-1 and to human embryonic kidney (HEK) 293 cells stably transfected with the CC CKR 2 A or B, using both radioligand binding studies and biotinylated MCP-1 and FACS analysis.

2. To examine the increase in intracellular calcium in response to MCP-1 using fura-2 labelled cells and to explore the source of this calcium rise with the aid of various calcium influx and calcium mobilisation inhibitors in THP-1 cells compared to the transfected cells and human blood derived monocytes and to identify the possible role for PLC by studying IP_3 generation.

3. To demonstrate the activation of PI 3-kinase by MCP-1 in THP-1 cells compared with the transfected cells by monitoring the production of PI 3-kinase products and to identify whether the PTK/SH2 -coupled PI 3-kinase and/or the G-protein -coupled PI 3-kinase are activated following MCP-1 stimulation using both a whole cell assay and an *in vitro* lipid kinase assay.

4. To determine whether MCP-1 induces tyrosine phosphorylation of proteins in THP-1 cells and the MCP-1 receptor transfected cells and to identify the role of these proteins in PI 3-kinase activation using Western blotting techniques.

5. To study the activation of various functional responses in monocytes following MCP-1 stimulation including chemotaxis, adhesion molecule upregulation, superoxide release and low affinity IgE receptor expression and to try to identify a possible role for the signal transduction pathways activated by MCP-1.

SECTION 2 : MATERIALS AND METHODS

2.1 Materials

Material	Source
β-glycerophosphate	Sigma, Poole, U.K.
[^{125}I]-MCP-1	Gift from Dr. M. Needham, Zeneca, U.K.
[^{32}P]-γATP	Dupont NEN, U.S.A.
[^{32}P]-orthophosphoric acid	Dupont NEN, U.S.A.
[^{3}H] PtdIns(4)P	Amersham International, Amersham, U.K.
[^{3}H] PtdIns(4,5)P_2	Amersham International, Amersham, U.K.
[^{3}H]-thymidine	Amersham International, Amersham, U.K.
'Immunolyse' blood lysing kit	Coulter, U.S.A.
2-mercaptoethanol	Sigma, Poole, U.K.
5/8μm PVPF chemotaxis membrane	Costar, High Wycombe, U.K.
Adenosine triphosphate	Sigma, Poole, U.K.
Ammonium persulphate	BDH, Poole, U.K.
anti-CD11b-FITC	Sigma, Poole, U.K.
anti-CD11c-FITC	Sigma, Poole, U.K.
anti-CD14-FITC	Sigma, Poole, U.K.
anti-CD23-FITC	Biosource International,
anti-IgG_1-FITC	Sigma, Poole, U.K.
anti-IgG_{2A}-FITC	Sigma, Poole, U.K.

anti-IgG-FITC	Sigma, Poole, U.K.
anti-VLA-4-FITC	Sigma, Poole, U.K.
Biorad protein reagent	Biorad, U.K.
Bisacrylamide solid	Sigma, Poole, U.K.
Bovine serum albumin	Sigma, Poole, U.K.
Bromophenol blue	BDH, Poole, U.K.
C5a	Sigma, Poole, U.K.
Calcium chloride	Sigma, Poole, U.K.
Chloroform	Fisons, Loughborough, U.K.
Coomassie blue	Sigma, Poole, U.K.
Cytochrome C	Sigma, Poole, U.K.
di-ammoniumhydrogen orthophosphate	Fisons, Loughborough, U.K.
Diff-Quik stain	Browne, Newbury, U.K.
Digitonin	BDH, Poole, U.K.
Dimethyl sulphoxide	Sigma, Poole, U.K.
Durapore filter plates	Millipore, Watford, U.K.
Econazole	Sigma, Poole, U.K.
EDTA	Sigma, Poole, U.K.
EGTA	Sigma, Poole, U.K.
Enhanced Chemiluminescence reagent	Amersham International, Amersham, U.K.
Eotaxin	Peprotech, Rocky Hill, USA.
Epidermal growth factor	Sigma, Poole, U.K.
Ethyl formate	Fisons, Loughborough, U.K.
Ferricytochrome C	Sigma, Poole, U.K.
Flo-scint IV scintillation fluid	Canberra Packard, U.K.

fMLP	Sigma, Poole, U.K.
Foetal bovine serum	Gibco BRL, Paisley, U.K.
Folch Lipids	Sigma, Poole, U.K.
Fura-2/AM	Calbiochem, U.K.
Geneticin (G418)	Gibco BRL, Paisley, U.K.
GF/A filter paper	Whatman, U.K.
Glacial acetic acid	Fisons, Loughborough, U.K.
Glutamine	Gibco BRL, Paisley, U.K.
Glycerol	Sigma, Poole, U.K.
Goat anti-mouse peroxidase conjugate	Gift from Dr. M. Welham, Bath, U.K.
Goat anti-rabbit peroxidase conjugate	Gift from Dr. M. Welham, Bath, U.K.
Hank's balanced salt solution	Gibco BRL, Paisley, U.K.
HEK 293 CC CKR 2A and 2B transfected cells	Gift from Dr. V. Schweickart, ICOS Corp., Washington, USA.
Heparin	C.P. Pharmaceuticals Ltd., Wrexham, U.K.
Hepes (1M liquid)	Gibco BRL, Paisley, U.K.
Hepes solid	Sigma, Poole, U.K.
Horseradish peroxidase	Sigma, Poole, U.K.
Hydrochloric acid	BDH, Poole, U.K.
Hydrogen peroxide	Sigma, Poole, U.K.
IL-3	R+D Systems, Abingdon, U.K.
IL-8	Gift from Dr. I. J. D. Lindley, Sandoz Research Institute, Austria.
Iodine	Sigma, Poole, U.K.
Iodoacetamide	Sigma, Poole, U.K.
Ionomycin	Sigma, Poole, U.K.

IP_3 assay kit	Amersham International, Amersham, U.K.
Leupeptin	Sigma, Poole, U.K.
Lithium chloride	Sigma, Poole, U.K.
Lymphoprep	Nycomed, Birmingham, U.K.
Magnesium chloride	Sigma, Poole, U.K.
Manganese chloride	Sigma, Poole, U.K.
MCP-1	Peprotech, Rocky Hill, USA.
MCP-1 Fluorkine kit	R+D Systems, Abingdon, U.K.
MCP-3	Peprotech, Rocky Hill. USA.
MEM medium	Gibco BRL, Paisley, U.K.
MEM non-essential amino acids	Gibco BRL, Paisley, U.K.
Methanol	Fisons, Poole, U.K.
Methylamine	Fisons, Loughborough, U.K.
MIP-1α	Peprotech, Rocky Hill, USA.
Molecular weight markers	Gibco BRL, Paisley, U.K.
Monoclonal MCP-1 antibody	R+D systems, Abingdon, U.K.
N-butanol	BDH, Poole, U.K.
Nickel chloride	Sigma, Poole, U.K.
NP40	Fisons, Poole, U.K.
Optiphase Scintillation fluid	Canberra Packard, U.K.
Orthophosphoric acid	Fisons, Loughborough, U.K.
Ovalbumin	Sigma, Poole, U.K.
p70 S6 kinase antibody	Santa Cruz, U.S.A.
p85 (α-isoform) antibody	Gift from Dr. D. Cantrell, I.C.R.F., U.K.
Penicillin / Streptomycin	Gibco BRL, Paisley, U.K.
Pepstatin	Sigma, Poole, U.K.

Perchloric acid	Fisons, Loughborough, U.K.
Pertussis Toxin	Gibco BRL, Paisley, U.K.
Petroleum Ether	BDH, Poole, U.K.
Phosphate buffered saline	Gibco BRL, Paisley, U.K.
Phosphate free DMEM	Gibco BRL, Paisley, U.K.
Phosphatidylinositol	Sigma, Poole, U.K.
Phosphatidylserine	Sigma, Poole, U.K.
Phosphotyrosine antibody (4G10)	Gift from Dr. M. Welham, Bath, U.K.
Phosphotyrosine antibody (PY20)	Sigma, Poole, U.K.
Plasticware (tissue culture grade)	Nunc, U.K.
PMA	Sigma, Poole, U.K.
PMSF	Sigma, Poole, U.K.
Potassium hydroxide	Fisons, Poole, U.K.
Potassium oxalate	Sigma, Poole, U.K.
Propan-1-ol	Fisons, Loughborough, U.K.
Propridium Iodide	Sigma, Poole, U.K.
Protein G beads	Sigma, Poole, U.K.
RANTES	Gift from Dr. T. Wells, GIMB, Geneva, Switzerland.
Ro31-8220-002	Gift from Dr. J.S. Nixon, Roche, U.K.
RPMI 1640	Gibco BRL, Paisley, U.K.
Scopoletin	Sigma, Poole, U.K.
Sodium azide	Sigma, Poole, U.K.
Sodium chloride	Sigma, Poole, U.K.
Sodium citrate	BDH, Poole, U.K.
Sodium dodecyl sulphate	Sigma, Poole, U.K.

Sodium fluoride	Sigma, Poole, U.K.
Sodium hydroxide	Fisons, Loughborough. U.K.
Sodium orthovanadate	Sigma, Poole, U.K.
Sodium pyruvate	Gibco BRL, Paisley, U.K.
Sulphuric acid	Fisons, Loughbrough, U.K.
Superoxide dismutase	Sigma, Poole, U.K.
TCA	Sigma, Poole, U.K.
TEMED	Sigma, Poole, U.K.
Tetrabutylammoniumhydrogen sulphate	Sigma, Poole, U.K.
THP-1 cells	ECCAC, Salisbury, U.K.
Tris base	Sigma, Poole, U.K.
Tris HCl	Sigma, Poole, U.K.
Tween 20	Sigma, Poole, U.K.
U73122/U73343	Affinity Laboratories,
Universal Indicator	Sigma, Poole, U.K.
Untransfected HEK 293 cells	ECACC, Salisbury, U.K.
Uridine triphosphate	Sigma, Poole, U.K.
Versene	Gibco BRL, Paisley, U.K.
Versene	Gibco BRL, Paisley, U.K.
Wortmannin	Sigma, Poole, U.K.
X-OMAT film	Amersham International, Amersham, U.K.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 THP-1 cells

THP-1 cells were cultured in RPMI 1640 medium (appendix 1). They were maintained at 37^0C in 95% O_2 / 5% CO_2 and passaged when they reached a concentration of between $5x10^5$ - $1x10^6$ cells/ml.

2.2.1.2 HEK 293 cells

HEK 293 cells transfected with either the MCP-1 type A or the MCP-1 type B receptor were obtained from ICOS Corp., USA.

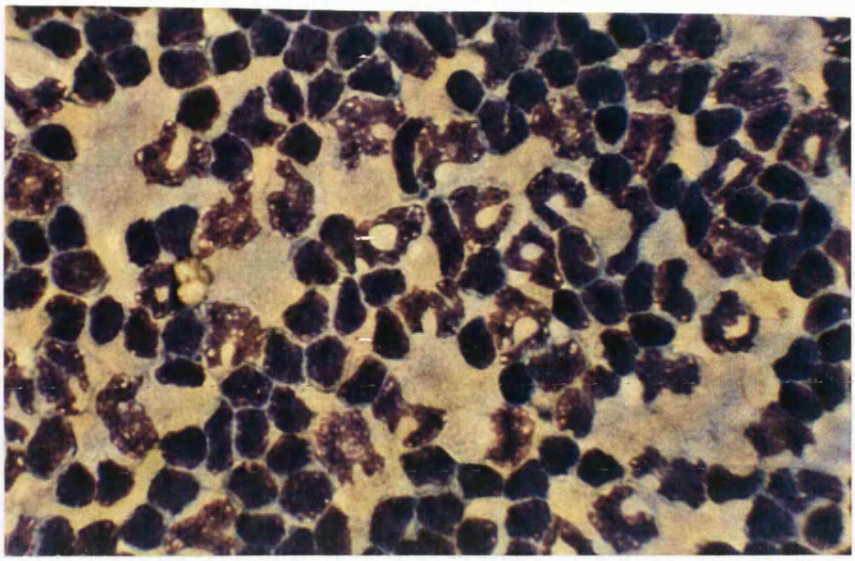
The transfected/untransfected control cells were cultured in T75 Nunc flasks in Modified Eagles medium (MEM) (appendix 1). The cells were dissociated from the flask using 3ml Versene (buffered EDTA solution) and washed in medium prior to passaging. The cells were passaged when confluent, i.e. 1 : 5 every seven days.

Prior to all experiments the HEK 293 cells were dissociated from the flask using 3 ml of Versene.

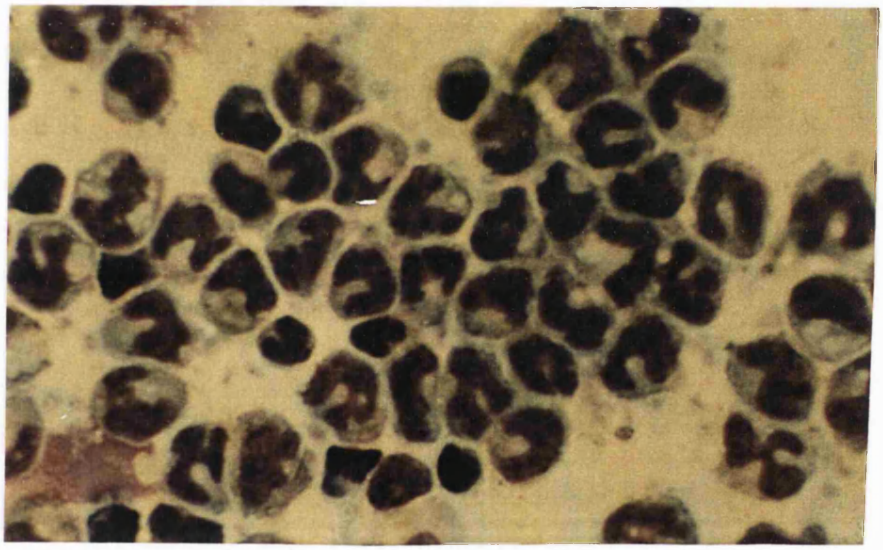
2.2.1.3 Monocyte preparation

Human venous blood from healthy individuals was collected into either 10 Units of heparin/ml blood or 3.8% citrate at 1ml per 9ml of blood. This was centrifuged at 800g for 6 minutes and the plasma aspirated off. The packed cell volume was diluted 1:1 with RPMI 1640 and layered 2:1 onto Lymphoprep (specific gravity 1.077). This was centrifuged at 400g for 30 minutes at room temperature without a brake.

a)



b)



Photograph 2.1 - Cytospin preparations of monocyte populations a) before and b) after plastic adherence. The PBMC's were purified as given in section 2.2.1.3 and then the monocytes isolated by plastic adherence. Samples of the preparations before and after this adherence step were prepared using a Shandon cytospin 3 and the slides stained with Diff-Quick stain. A large number of the cells in photograph a) can be seen as lymphocytes, small cells with a large dense nucleus and scanty surrounding cytoplasm. In photograph b) there are very few lymphocytes with the majority of the cells being monocytes, with horse-shoe shaped nuclei and an abundant cytoplasm (Magnification x 40).

The peripheral blood mononuclear cell (PBMC) layer between the RPMI and the Lymphoprep was removed and washed with a three fold excess of RPMI 1640 and centrifuged at 500g for 10 minutes. The PBMC's were then plated out into petri dishes in RPMI 1640 with 10% foetal calf serum (FCS) at $\approx 2x10^7$ cells/dish and left at 37°C for one hour in 95% O_2 / 5%CO_2. The non-adherent cells were removed and discarded and the adhered monocytes were gently scraped off the petri dish using a plastic cell scraper and washed once with RPMI 1640 and used in the experiment. A cytospin of the cell suspension was carried out to determine the purity of the population (Photograph 2.1a and b). Purity was always between 90-95%.

2.2.2 Radioligand binding

A 96 well Durapore filter plate (0.65μm pore size) was washed with 200μl of phosphate buffered saline (PBS) containing 10mg/ml bovine serum albumin (BSA) and dried at 37°C to reduce non-specific binding to the filters. The HEK 293 cells and the THP-1 cells were washed twice and resuspended in binding buffer (appendix 2) at $5x10^6$ cells/ml and $1x10^7$ cells/ml respectively. 50μl of the competing ligand (0.1-30nM) or binding buffer was added to the appropriate wells followed by 50μl of 400pM ^{125}I-MCP-1 (total counts ≈ 55 000cpm, stock specific activity 2000Ci/mmol, 100Ci/ml). The incubation was started by the addition of 100μl of THP-1 cells ($1x10^6$/well) or HEK 293 cells ($5x10^5$ /well) and left at 4°C for 90 minutes. Following incubation, the plate was washed twice with ice cold binding buffer containing 0.5M NaCl using a Multiscreen vacuum manifold for 96 well Durapore plates from Millipore. The plate was blotted on paper towel and the filters punched out using Multiscreen multiple punch apparatus and disposable punch tips. Each filter was then counted for two minutes on an LKB Wallac 1277 Gammamaster automatic gamma counter.

Non-specific binding to the filters was determined by incubating ^{125}I-MCP-1 in the absence of cells. This value was then subtracted from the binding data obtained in the presence of cells. All the binding data was normalised by expressing it as a percentage of the total binding. The total binding was defined as the radioactivity bound to the cells in the absence of any competing ligand.

In order to be able to calculate the dissociation constant for MCP-1 binding (Kd) and the number of receptors per cell (B_{MAX}), a Scatchard transformation was performed, in which the ratio of the number of moles of bound ligand/free ligand was plotted against the number of moles of bound ligand (Scatchard, 1949). This transformation was carried out using the Grafit binding program (Leatherbarrow, 1992). This Scatchard plot also determined the number of receptor types present on the cell. A straight line indicates a single receptor type. This straight line has a slope of 1/Kd and an intercept with the x-axis of B_{MAX}. The number of receptors per cell can be directly calculated from the B_{MAX} using equation 1.

Equation 1

$$\text{Number of receptors per cell} = \frac{(B_{MAX} / 5000)}{\text{cell number}} \times N_A$$

Where B_{MAX} = intercept with the x-axis

5000 = dilution factor (B_{MAX} is given as moles/l but only 200µl/well used in the assay)

N_A = Avogadro's number = 6.02×10^{23} moles^{-1}

In some cases the Scatchard plot departs significantly from linearity. A curvilinear plot indicates the presence of more than one receptor type, each with differing Kd values.

2.2.3 Binding by FACS analysis

Recently, R+D Systems have developed a binding assay for MCP-1 which utilises biotinylated MCP-1. Biotinylated MCP-1 is incubated with the cells and binds to receptors on the cell surface. The cells are then directly incubated with fluoresceinated-avidin which attaches to the receptor-bound biotinylated MCP-1. Cells expressing the receptors become fluorescently labelled and the intensity of this fluorescence staining is directly proportional to the density of receptors present on the cell surface. The fluorescence is then determined using a fluorescence activated cell sorter (FACS).

FACS is a technique in which cells flow in single file in the centre of a fluid stream through a fine orifice at rates of up to 2-3 thousand cells per second (Watson, 1991). A light source illuminates the particle stream and detectors measure both the light scattered by the cell, giving an indication of cell size and the fluorescence intensity emitted from the fluorescent probe with which the cells have been labelled. The FACS is also able to separate, or sort, the cells into distinct sub-populations using various parameters such as fluorescence intensity or size. FACS analysis of binding has one main advantage and that is its sensitivity. This makes it possible to detect very low numbers of receptors i.e. a few thousand per cell.

2.2.3.1 Assay procedure

HEK 293 cells and THP-1 cells were washed twice and resuspended at a concentration of $4x10^6$ cells/ml in PBS. 25µl of the cell suspension was added to polypropylene FACS tubes and incubated with 10µl of biotinylated MCP-1 (stock = 224nM) or negative control for 60 minutes at 4°C.

To check the specificity of the MCP-1 binding, both non-biotinylated MCP-1 and an anti-MCP-1 antibody were used. The non-biotinylated MCP-1 was added at a final concentration of 1μM, prior to the biotinylated MCP-1 and the samples were incubated for 60 minutes at 4°C.

For the anti-MCP-1 antibody samples, 10μl of the biotinylated MCP-1 was incubated with 20μl of the 2mg/ml stock anti-MCP-1 antibody for 15 minutes at room temperature prior to its addition to the cell suspensions. Also, to block Fc-mediated reactions, the cell suspensions were incubated with 0.1mg/ml anti-mouse IgG antibody at room temperature for 15 minutes prior to the addition of the biotinylated MCP-1/anti-MCP-1 antibody complex. The samples were incubated for 60 minutes at 4°C.

Following the 60 minute incubation at 4°C, 10μl of avidin-fluoroscein isothiocyanate (FITC) conjugate (f:p ratio 5:1) was added to the suspensions and incubated for a further 30 minutes at 4°C. After the second incubation, the cell suspensions were washed twice with a working solution of the RDF1 wash buffer provided with the kit containing 0.5M NaCl and then resuspended in RDF1 wash buffer without NaCl. The amount of binding to the live cells (as defined by the lack of uptake of propridium iodide) was analysed using a Becton Dickinson FACS Vantage fluorescence activated cell sorter, collecting 10 000 events for each sample.

2.2.4 Measurement of $[Ca^{2+}]_i$

The measurement of $[Ca^{2+}]_i$ has been made possible by the development of a number of calcium sensitive dyes including fura-2 (review (Walt *et al.* 1987)). Fura-2 can be non-disruptively loaded into the cells as the acetoxymethyl ester (AM) since the esterified fura-2 is uncharged and hydrophobic and can cross the plasma membrane.

Once in the cells, endogenous esterases release the free fura-2 which cannot permeate out of the cell. The fluorescence excitation maximum of fura-2 shifts to a lower wavelength upon calcium binding, without any change in the emission maximum. Thus, fura-2 can be used as a dual excitation indicator. The excitation maximum for calcium-free and calcium-bound fura-2 are monitored at 380nm and 340nm respectively. The emission maximum is monitored at 510nm. Fura-2 also has an isofluorescence, or isobestic, point at 360nm which can be used to monitor calcium-independent fluorescence. For example, manganese can be used to monitor influx pathways as it binds to fura-2 causing a quenching of the fluorescence at 360nm.

2.2.4.1 $[Ca^{2+}]_i$ measurements in a cell population

2.2.4.1.1 Loading cells with fura-2/AM

The cells were pelleted by centrifugation and resuspended in Hanks balanced salt solution (HBSS) (without calcium (Ca^{2+})/magnesium (Mg^{2+})) containing 0.1% BSA (HBSS/BSA) at between $1x10^6$ - $1x10^7$ cells/ml. The HEK 293 cells and THP-1 cells were then incubated with 5μM fura-2/AM at 37°C for 40 minutes and 30 minutes respectively. Subsequently, the cells were washed twice and resuspended at $1x10^6$ cells/ml in HBSS/BSA. Human monocytes were purified as given in section 2.2.1.3 and loaded with fura-2/AM as described for HEK 293 cells.

2.2.4.1.2 $[Ca^{2+}]_i$ measurement

The cells were aliquoted into a 2ml quartz cuvette at a final concentration of $3x10^5$ cells/ml for the THP-1 cells and $1x10^6$ cells/ml for the HEK 293 cells and for the human monocytes, if enough were obtained from the purification procedure. 1mM Ca^{2+} and 1mM Mg^{2+} was added to the cell suspension in the cuvette and equilibrated in the 37°C chamber in the spectrofluorimeter for 4 minutes. The cell suspensions were constantly stirred using a small magnetic stirrer bar. A basal intracellular

calcium measurement was taken for 40 seconds prior to the addition of the agonist. The response was monitored for at least 60 seconds and detected using dual excitation wavelengths of 340nm and 380nm and a single emission wavelength of 510nm on a dual excitation/dual emission spectrofluorimeter from Photon Technology International.

For the manganese influx experiments, the cells were loaded and maintained in HBSS/BSA with 100μM Ca^{2+} and then pelleted immediately prior to use and resuspended and treated as given above. The changes were then monitored at an excitation wavelength of 360nM and an emission wavelength of 510nm.

The administration time of any compounds used was as given in the appropriate figure legends during the 4 minutes pre-incubation period.

2.2.4.1.3 Fluorimeter calibration

Calibration of the system was achieved by monitoring the fluorescence changes in the cell suspensions after the addition of 0.16μg/ml digitonin followed by the addition of 40mM sodium hydroxide (NaOH) and 4mM [ethylenebis(oxyethylenenitrilo)tetra-acetic] acid (EGTA) 30 seconds later. Conversion of the change in intracellular calcium from the ratio of the fluorescence at the two wavelengths to a nM calcium concentration was then determined as defined by Grynkiewicz *et. al.* (Grynkiewicz *et al.* 1985). In brief, the equation is as follows:

Equation 2

$$[Ca^{2+}]_i = Kd \frac{R-R_{min}}{R_{max}-R} \frac{S_{f2}}{S_{b2}}$$

Where, Kd = effective dissociation constant for fura-2 (2.24×10^{-7} M)

$R = Ca^{2+}$ bound / Ca^{2+} free fluorescence ratio

R_{min} = fluorescence ratio with zero calcium

R_{max} = fluorescence ratio under saturating calcium conditions

S_{f2}/S_{b2} = ratio of fluorescence values for Ca^{2+} bound / Ca^{2+} free indicator measured at the wavelength used to monitor Ca^{2+} - free indicator (denominator wavelength of R) i.e. at 380nm.

2.2.4.2 $[Ca^{2+}]_i$ measurements on a small population of adherent cells

The $[Ca^{2+}]_i$ changes in HEK 293 cells were also studied at the single cell level. This was performed by growing the cells on 22mm diameter glass cover slips. When sub-confluent, the cells were loaded with 5μM fura-2/AM at 37°C for 40minutes in HBSS (with 1mM Ca^{2+}/Mg^{2+}) / BSA. The adherent cells were then washed twice and placed in a 37°C chamber with 1ml HBSS (with Ca^{2+}/Mg^{2+})/ BSA. This chamber was positioned on a fluorescence microscope from Photon Technology International and a population of approximately 30 cells was selected by adjusting the field of view. Using a x 40 oil immersion objective, the fluorescence changes were monitored for 40 seconds before and 60 seconds after agonist addition. The results were expressed as the fluorescence ratio at 340/380nm wavelengths.

2.2.5 Inositol (1,4,5) trisphosphate (IP_3) measurement

The measurement of IP_3 can be performed by two different techniques. The first relies on the labelling of cells with [^{3}H]-inositol and the separation of IP_3 isomers by HPLC (Meek, 1986). The main drawback with this method is the assumption that all the phosphoinositide pools have been labelled to equilibrium, but this is rarely

achieved. The second method was first developed by Challiss *et al.* in 1988 (Challiss *et al.* 1988) and has subsequently been optimised and marketed by Amersham International. This assay is a competition assay using a bovine adrenal cortex membrane preparation as a binding protein. [^{3}H]-IP_3 is incubated with this binding protein in the presence of non-labelled IP_3/samples and the IP_3 concentration in the samples calculated from a standard curve. The radioreceptor approach is both faster and more suitable for analysis of large sample numbers than the isotopic method. Also, because it does not rely on cell labelling, it is both more accurate and more sensitive as possible agonist-induced changes in the specific radioactivity of IP_3, which can occur in the isotopic method, do not apply.

2.2.5.1 Sample preparation

The HEK 293 cells and the THP-1 cells were washed once in HBSS with 0.1% BSA and resuspended at a concentration of $2x10^7$ cells/ml in HBSS / BSA containing 1mM Ca^{2+} and 1mM Mg^{2+}. 500µl aliquots were put into 1.5ml Eppendorf tubes and, following 10 minutes equilibration at 37°C, were stimulated with 50µl of agonist for various times. The reaction was quenched by the addition of 0.22 volumes of ice cold 20% (v/v) perchloric acid. After 20 minutes incubation on ice, the proteins were pelleted at 10 000g for 5 minutes. The supernatants were titrated to pH 7.5 with 1.5M potassium hydroxide (KOH) containing 60mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (Hepes) buffer in the presence of 1µl universal indicator.

2.2.5.2 Detection of IP_3

The IP_3 levels in these supernatants were assayed immediately, using the IP_3 detection kit from Amersham International. In brief, the sample was incubated with 0.07µCi/ml [^{3}H]-IP_3 and bovine adrenal membrane binding protein for 15 minutes on ice in parallel with IP_3 standards ranging from 0.19-25pmol / tube in H_2O (assay sensitivity 0.1 pmol/tube) . The samples were then centrifuged and the pellets

resuspended in 0.15M sodium hydroxide to release the all of the IP_3 bound to the receptor. After 10 minutes incubation at room temperature the samples were decanted into scintillation vials and 4ml of scintillation fluid was added. The tubes were read on an LKB Wallac 1215 Rackbeta liquid scintillation counter for 4 minutes each. The results were calculated using linear regression from the standard curve, performed in parallel with the samples. A typical standard curve is shown in figure 2.1.

2.2.6 PI 3-kinase activity

There are two separate methods for measuring the agonist-induced activation of PI 3-kinase. These are the measurement of the accumulation of D-3 phosphatidylinositol lipids in intact cells and the immunoprecipitation of PI 3-kinase using an antibody to the p85 subunit followed by an *in vitro* lipid kinase assay of the immunoprecipitated PI 3-kinase. These two assays differ in the PI 3-kinase activity they measure. The former accounts for all the PI 3-kinase activities that are activated in the cells. In contrast, the *in vitro* lipid kinase assay only measures the activation of the PTK/SH2 - coupled PI 3-kinase, immunoprecipitated by the p85 antibody.

2.2.6.1 Accumulation of D-3 phosphatidylinositol lipids in intact cells

2.2.6.1.1 Sample preparation

The cells were washed three times in phosphate-free Dulbeccos Modified Eagles medium (DMEM) with 10 minute incubations at 37°C before each spin of 400g for 5 minutes. The cells were subsequently resuspended in 10ml phosphate-free DMEM containing 10% dialysed foetal calf serum and 20mM HEPES at $1x10^7$ cells/ml . THP-1 cells were incubated with 1mCi of [^{32}P]-orthophosphoric acid (100μCi/ml, 185MBq) at 37°C for 4 hours. The HEK 293 cells were incubated with 0.5mCi [^{32}P]-orthophosphoric acid at 37°C for 1 hour.

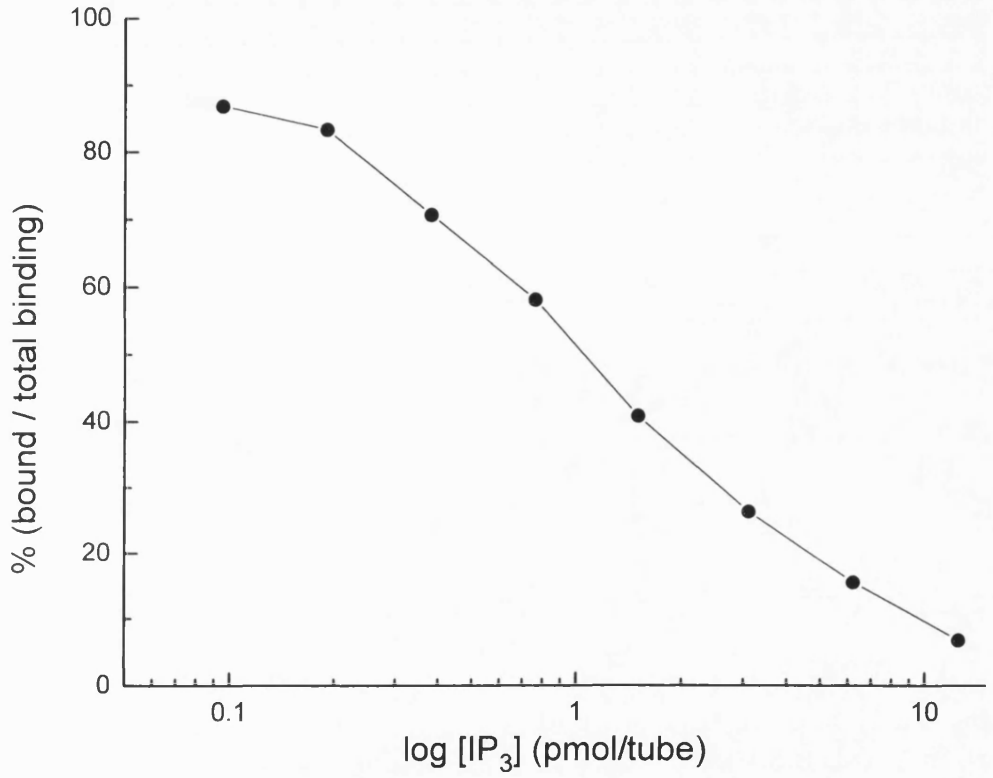


Figure 2.1 - A typical standard curve for the measurement of IP_3.
Non-specific binding was defined as the total binding in the presence of excess unlabelled IP_3 (0.1nmoles). This was subtracted from the binding data and the percentage of bound/total binding was plotted against log [IP_3]. The total binding was defined as the amount of radioactivity in the absence of any competing ligand. Data is mean of duplicate samples.

After incubation, the cells were washed three times in phosphate-free DMEM and resuspended in RPMI 1640. $1x10^7$ [^{32}P]-labelled cells were aliquoted in a volume of 120µl into 1.5ml Eppendorf tubes and equilibrated at 37°C for 10 minutes. The cells were stimulated with 12µl of agonist or vehicle for the appropriate times and the reaction quenched by the addition of 700µl of ice cold chloroform/methanol/water (32.6%/65.3%/2.1%) according to the method described by Jackson *et al.* (Jackson *et al.* 1992). The samples were immediately put on ice. 600µl of chloroform containing 10µg of Folch lipids as a carrier protein were then added along with 100µl of 2.4M hydrochloric acid (HCl), 5mM tetrabutylammoniumhydrogen sulphate. The extraction mixtures were vortexed and centrifuged at 3 000g for 5 minutes. The lower phase of the extraction mixture was subsequently removed and added to 400µl of 1M HCL, 5mM ethylenediaminetetraacetic acid (EDTA). The mixtures were again vortexed and centrifuged at 3000g for 5 minutes. The lower phase was removed and dried *in vacuo* using a Savant SpeediVac . When dried down, 1ml of 25% (w/v) methylamine/methanol/N-butanol (4/4/1) was added to the residue and, after vortexing, the samples were incubated in a 53°C water bath for 40minutes. This deacylation procedure renders the glycerophosphorylinositol derivatives of PtdIns(3)P (GroPIns(3)P), PtdIns (3,4)P_2 (GroPIns(3,4)P_2) and PtdIns(3,4,5)P_3 (GroPIns(3,4,5)P_3) water soluble. The samples were then cooled for 1 minute on ice and dried *in vacuo*. Finally, 500µl of water and 600µl of N-butanol/40-60% petroleum ether/ethyl formate (20/4/1) were added. The samples were vortexed and centrifuged at 750g for 30 seconds. The upper phase was removed and discarded and the lower phase was dried *in vacuo*. The samples were then stored at -20°C until analysis.

2.2.6.1.2 <u>HPLC analysis of samples</u>

Anion exchange high performance liquid chromatography (HPLC) was used to analyse the lipid content of the samples using a water and phosphate buffer gradient (Stephens *et al.*. 1989). The phosphate buffer was 1.25M *di*-ammoniumhydrogen

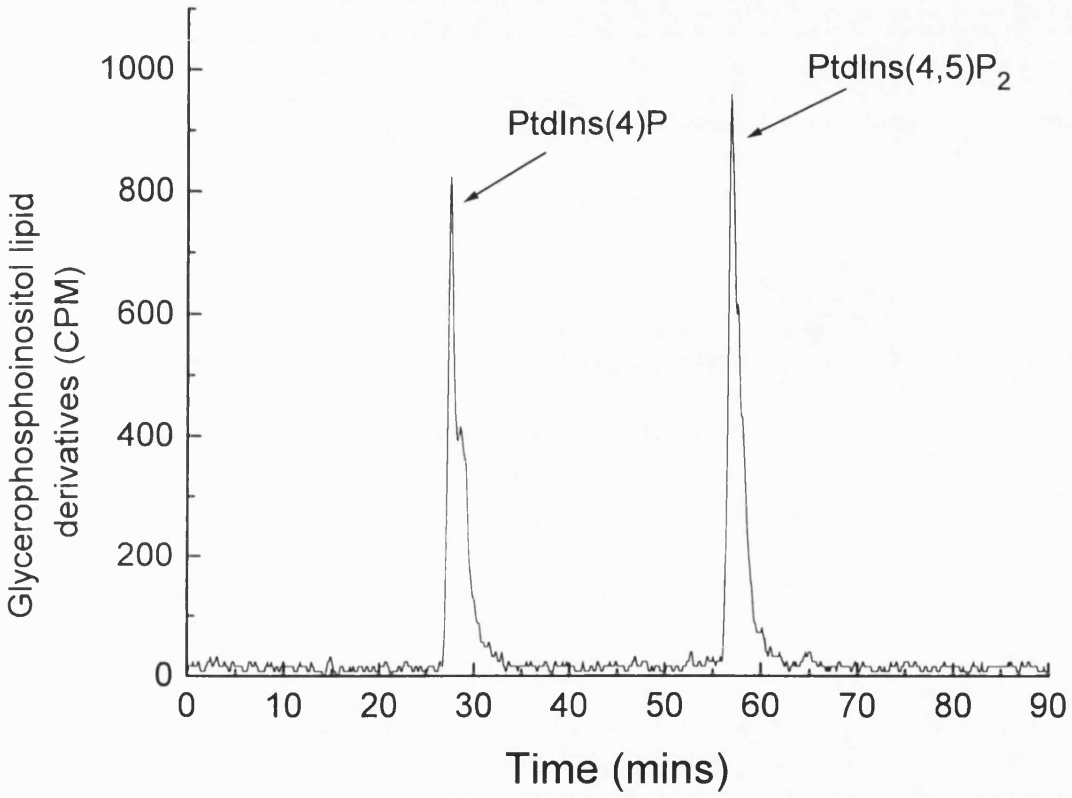


Figure 2.2 - An example of a trace showing the retention times of the [^{3}H]PtdIns(4)P and [^{3}H]PtdIns(4,5)P_2 standards used in the analysis of PI 3-kinase products. The elution times of the 3-phosphorylated were compared to previous results obtained by Dr. Stephen Ward and previously published data (Stephens *et al.* 1991).

orthophosphate ($(NH_4)_2HPO_4$) with the pH adjusted to 3.8 using orthophosphoric acid. The samples were resuspended in 120µl of water and injected onto a Partisphere SAX column. The eluant was detected using a Canberra Packard A-500 Flo-One on-line *beta* radiodetector where it was mixed in a ratio of 1:3 with Flo-Scint IV scintillation cocktail, according to manufacturers specifications. The results were analysed on a FLO-one data program. The retention times were compared to standards of $[H^3]$PtdIns(4)P and $[H^3]$PtdIns(4,5)P_2. An example of the standards trace is seen in figure 2.2. The identity of the various peaks obtained using this separation technique has been defined previously (Stephens *et al.* 1991). The PtdIns(3)P elution time was compared to that determined by Dr. Stephen Ward by immunoprecipitating PI 3-kinase using an anti-p85 antibody and performing an *in vitro* lipid kinase assay. The PtdIns(3)P was isolated by TLC and then extracted from the TLC plate and its elution time on the HPLC monitored (personal communication) (Ward *et al.* 1995). The elution times of the other 3-phosphorylated lipids were compared to those quoted in the literature (Stephens *et al.* 1991) (Traynor-Kaplan *et al.* 1988) (Cheatham *et al.* 1994). The levels of PtdIns were used as an internal standard to confirm that each sample contained a similar amount of radioactivity as the PtdIns pool should not vary upon agonist stimulation.

2.2.6.2 Immunoprecipitation and *in vitro* lipid kinase assay

2.2.6.2.1 Coupling the antibody to protein G sepharose beads

The protein G sepharose beads from Sigma were provided in methanol and were therefore washed three times with 1 ml of PBS and then resuspended as a 50% suspension in PBS. 500µl aliquots were stored at 4°C until required.

50µl of anti-p85 antibody was added to 500µl of 50% bead suspension and the volume increased to 1ml with PBS. The suspension was rotated for two hours at room temperature. Following rotation, the bead suspension was washed three times with

1ml of PBS and again resuspended as a 50% suspension by the addition of 250μl of PBS and stored at 4°C until required.

To confirm that the antibody had coupled to the beads, 10μl of the beads were denatured by heating to 90°C for 10 minutes in reducing sample buffer (appendix 5). The samples were then separated by a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (appendix 5), along with molecular weight markers, using Biorad Mini Protean II gel running apparatus. The gel was run at 75 volts whilst the samples traversed through the stacking gel and 175 volts whilst they traversed through the resolving gel. The gel was stained with Coomassie blue stain for 6 hours and subsequently destained for 24 hours (appendix 5) to detect the heavy and light chains of the antibody. See figure 2.3 for an example of the gel.

2.2.6.2.2 Immunoprecipitation of the PI 3-kinase.

This was performed as described by Ward *et al.* (Ward *et al.* 1992). HEK 293 cells and THP-1 cells were pelleted by centrifugation and washed twice with RPMI 1640 and resuspended in RPMI 1640 + 20mM Hepes at a concentration of $2x10^7$ cells/ml. 500μl aliquots of the cells were allowed to equilibrate at 37°C for 10 minutes and then stimulated with 50μl of agonist for the appropriate times. The reaction was stopped by the addition of equal volumes of ice cold lysis buffer (appendix 3). The samples were then rotated at 4°C for 15 minutes and the cell debris pelleted at 10 000g for 15 minutes at 4°C. 20μl of lysate was kept for a total kinase sample and the rest of the lysates were removed and added to 1.5ml Eppendorf tubes containing 20μl of protein G sepharose beads. The samples were then pre-cleared by rotating at 4°C for 60 minutes to remove any non-specific proteins that may bind to protein G-sepharose. The pre-cleared samples were spun at 10 000g for 5 minutes and the supernatants removed and added to 1.5ml Eppendorf tubes containing 20μl of the antibody-coupled protein G beads. These suspensions were rotated for 120 minutes at 4°C.

Subsequently, the immunoprecipitates were washed 3 times with lysis buffer, once with PBS, twice with 500mM lithium chloride (LiCl) (pH 7.4), once with water and once with lipid kinase buffer (appendix 4), spinning at 10 000g between each wash. After the final wash all the surplus kinase buffer was removed using a Hamilton syringe.

2.2.6.2.3 *In vitro* lipid kinase assay.

Each of the samples, including the total lysate sample, were resuspended in 40μl of lipid kinase buffer. 50μl of a lipid substrate mixture (1 mg of PtdIns and 1 mg of phosphatidylserine (PtdS) made up in 2ml of 25mM Hepes/1mM EDTA and dispersed by sonication in three 15 second bursts on ice) was added to the immunoprecipitates. The reaction was initiated by the addition of 5 μCi of [γ-^{32}P]-ATP (S.A. 3000Ci/mmol, 0.5mCi/ml, 18.5MBq) and 100μM ATP. The samples were incubated in a 25°C water bath for 15 minutes and the reaction quenched using 100μl 1M HCL and 200μl 1:1 chloroform:methanol. The samples were spun for 30 seconds at 10 000g and then the lower chloroform layer removed and dried *in vacuo*.

The dried samples were resuspended in 50μl chloroform and applied to a 1% oxalate sprayed thin layer chromatography (TLC) plate. The plate was placed in a TLC tank that had been equilibrated for at least 6 hours with propan-1-ol:2N glacial acetic acid (65:35 (v/v)) and lined with filter paper to ensure adequate vapour equilibration. The TLC was run for 15 hours.

Subsequently, the plate was air dried and exposed to iodine to detect the substrates and finally exposed to a film for 1-12 hours at -70°C. The film was developed using an RGII Fuji X-ray film processor. The total lysate sample contained not only PI 3-kinase but also other kinases such as PI 4-kinase. This provided a positive control for the *in vitro* kinase assay to confirm that the assay was working.

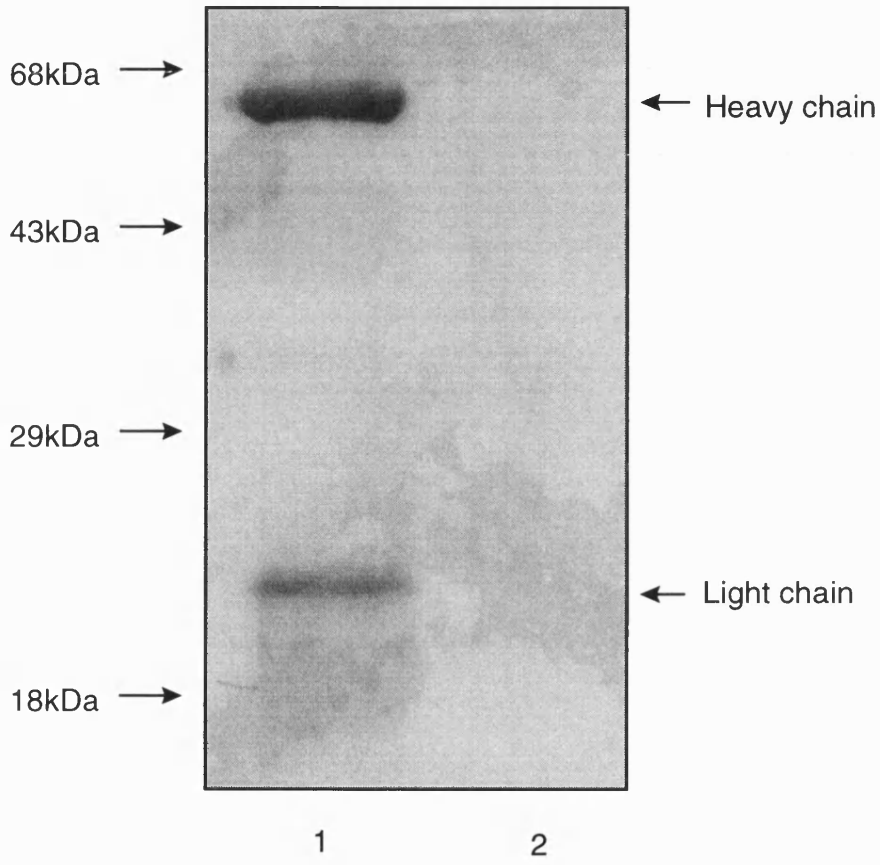
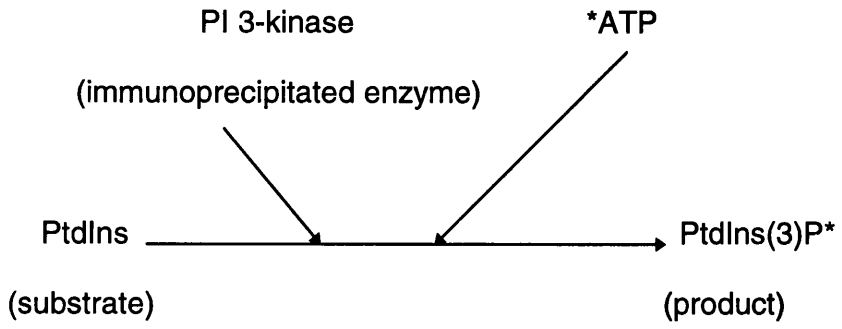


Figure 2.3 - Example of an SDS-PAGE gel to show coupling of the p85-antibody to the protein G sepharose beads. Lane 1 shows the p85 antibody coupled beads and lane 2 shows the control, uncoupled beads. The molecular weight markers are indicated by the arrows as are the heavy and light chains of the antibody.

Figure 2.4 - Diagram to show the reaction which takes place in the *in vitro* lipid kinase assay



The PtdIns(3)P product visualised by autoradiography was confirmed from previous experiments performed by Dr. Stephen Ward, in which the product was extracted from the TLC plate and analysed by HPLC (personal communication).

2.2.7 Detection of tyrosine phosphorylated proteins

2.2.7.1 Sample preparation

THP-1 cells and HEK 293 cells were washed twice with RPMI 1640 and resuspended at $2x10^7$ cells/ml in RPMI 1640 with 20mM Hepes. 500µl aliquots of cells were equilibrated at 37°C for 10 minutes prior to stimulation with 50µl of agonist for various times. The reaction was quenched by the addition of an equal volume of ice cold lysis buffer (appendix 3). The samples were rotated for 15 minutes at 4°C and then the cell debris pelleted at 10 000g for 15 minutes at 4°C. The supernatant was pre-cleared for 1 hour by rotating with 20µl of a 50% suspension of protein G-sepharose beads at 4°C. This removed any non-specific proteins which may have bound to protein G-sepharose.

The beads were spun down at 10 000g for 5 minutes at 4°C and then the tyrosine phosphorylated proteins in the supernatants were immunoprecipitated by rotating with 20µl of a 50% suspension of protein G beads coupled to PY20 anti-phosphotyrosine

antibody (coupling method as given for p85 antibody coupling in section 2.2.6.2.1) for 2 hours at 4°C. The immunoprecipitates were washed 5 times with lysis buffer and denatured by heating to 90°C for 10 minutes with 15µl reducing sample buffer (appendix 5).

The proteins were separated on a 7.5% SDS gel using Biorad Mini Protean II gel running apparatus, Western blotted and detected as given in section 2.2.9.

2.2.8 Phosphorylation of p70 S6 kinase

2.2.8.1 Sample preparation

THP-1 cells were depleted of serum in the culture medium for 24 hours prior to the sample preparation. The cells were then washed once and resuspended at $2x10^7$ cells/ml in RPMI 1640 with 20mM Hepes. 500µl aliquots of cells were equilibrated to 37°C for 10 minutes and then stimulated with 50µl of agonist for various times. The reaction was quenched by the addition of an equal volume of ice cold lysis buffer (appendix 3). The cell lysates were rotated for 15 minutes at 4°C and then the cell debris pelleted by centrifugation at 10 000g for 15 minutes at 4°C. The proteins were precipitated by the addition of 700µl of ice cold acetone to 500µl of the cell supernatant. The samples were left on ice for 15 minutes and then the proteins pelleted by centrifugation at 10 000g for 15 minutes at 4°C. The acetone was dried off *in vacuo* and the concentrated proteins denatured in 100µl of sample buffer (appendix 5) by heating to 90°C for 10 minutes.

The proteins were separated on a 15% SDS gel, containing an acrylamide/bisacrylamide mixture of 30%/0.08% respectively instead of 30%/0.8%, using the Hoefer SE400 'Sturdier' vertical slab gel unit. The proteins were Western blotted and detected as given in section 2.2.9.

2.2.9 SDS-PAGE and Western blotting

2.2.9.1 Separation of proteins

The proteins were separated on an SDS gel of the percentage specified in the sample preparation (sections 2.2.7 and 2.2.8)(appendix 5) in parallel with molecular weight markers following a method adapted from Laemmli (Laemmli, 1970). The gels were run at 75 volts whilst the samples traversed through the stacking gel and then 180 volts to the bottom of the resolving gel.

The separated proteins were transferred onto a nitrocellulose membrane by semi-dry transfer. This involved wetting the electrodes in semi-dry transfer buffer (appendix 5) and making a sandwich of 4 pieces of filter paper, membrane, gel and a further 4 pieces of filter paper, all soaked in semi-dry transfer buffer. The transfer was run at 0.8mA/cm^2 of membrane for 1 hour on Pharmacia-Biotech Multiphor II semi-dry transfer apparatus. The membrane was then stained with Ponceau S to check for transfer / even loading of the samples and the stain removed by washing the membrane for 2 minutes with water and for 10 minutes with Tyrodes buffered saline (TBS).

2.2.9.2 Development of the blot

The non-specific protein binding was blocked by overnight incubation of the membrane with blocking buffer (appendix 5) at room temperature. The membrane was washed once for 10 minutes with TBS and then incubated for 3 hours at room temperature with primary antibody specified in table 2.1, made up in 1:5 diluted blocking buffer.

Table 2.1 - Primary and secondary antibodies used for the development of tyrosine phosphorylation and p70 S6 kinase phosphorylation Western blots

Assay	Primary antibody	Secondary antibody
Protein tyrosine phosphorylation	0.1μg/ml 4G10 anti-phosphotyrosine antibody	1/10 000 goat anti-mouse horseradish peroxidase conjugate
p70 S6k phosphorylation	0.1μg/ml p70 S6k antibody	1/10 000 goat anti-rabbit horseradish peroxidase conjugate

The membrane was washed once with TBS (appendix 5), 3 times with TBS/NP40 and finally once with TBS, each for 10 minutes. The membrane was subsequently incubated for a further 90 minutes with 1/10 000 horseradish peroxidase conjugated secondary antibody specified in table 2.1, diluted in TBS/NP40. Again, the membrane was washed as given for the primary antibody with an extra TBS wash at the end and finally incubated with 10ml Enhanced Chemiluminescence (ECL) reagent for 1 minute. The membrane was exposed to a film for 30seconds - 30 minutes and the film developed using an RGII Fuji X-ray film developer.

2.2.10 Proliferation assay

The proliferation rate of cells can be determined by monitoring the incorporation of [^{3}H]-thymidine into the cell's DNA.

The HEK 293 cells were plated out in 96 well plates at $5x10^4$ cells/ml with 100µl per well and incubated at 37°C in 95% O_2 / 5% CO_2 for 40 hours. The cells were then serum starved for 4 hours prior to the reintroduction of serum at varying percentages (0, 1, 5 and 10%). Also, the effects of 1% FCS in the presence of varying concentrations of MCP-1 (1.25-60nM) or 60nM MCP-1 pre-incubated with 0.3mg/ml neutralising anti-MCP-1 antibody were tested. The cells were subsequently incubated for 48 hours, pulsing each well with 1µCi [^{3}H]-thymidine (S.A. 5Ci/mmol, 1mCi/ml, 37MBq) for the final 18 hours. The cells were harvested by washing with 5% TCA, water and finally methanol using a Skatron Combi cell harvester. The cells were collected onto Whatman GF/A filter paper and the filters were then submerged in 4ml of Optiphase scintillant and the samples counted on an LKB Wallac 1215 Betarack liquid scintillation counter for 1 minute per sample.

2.2.11 Chemotaxis

This method was modified from that described by Falk *et al.* (Falk *et al.* 1980). The monocytes were prepared as given in section 2.2.1.3 and resuspended in RPMI containing 0.1% BSA at a final concentration of $1.75x10^6$cells/ml. THP-1 cells were used at a concentration of $4x10^6$ cells/ml. 27µl of agonist was placed in the lower wells of the Boyden chamber (figure 2.5). The two chambers were then separated by a 5 or 8µm polyvinlypyrolidine-free (PVPF) membrane, orientated with the shiny side upwards, which was secured in place by a silicon gasket and the upper chamber. Prior to the addition of the cells, the chamber was incubated at 37°C for 10 minutes and then 40µl of the cell suspension was added to the upper wells.

For chemokinesis experiments the agonist was placed in both the lower and the upper wells. The chamber was then incubated at 37°C with 5% CO_2 for 90-120 minutes. Following incubation, the upper side, or shiny side, of the membrane was washed

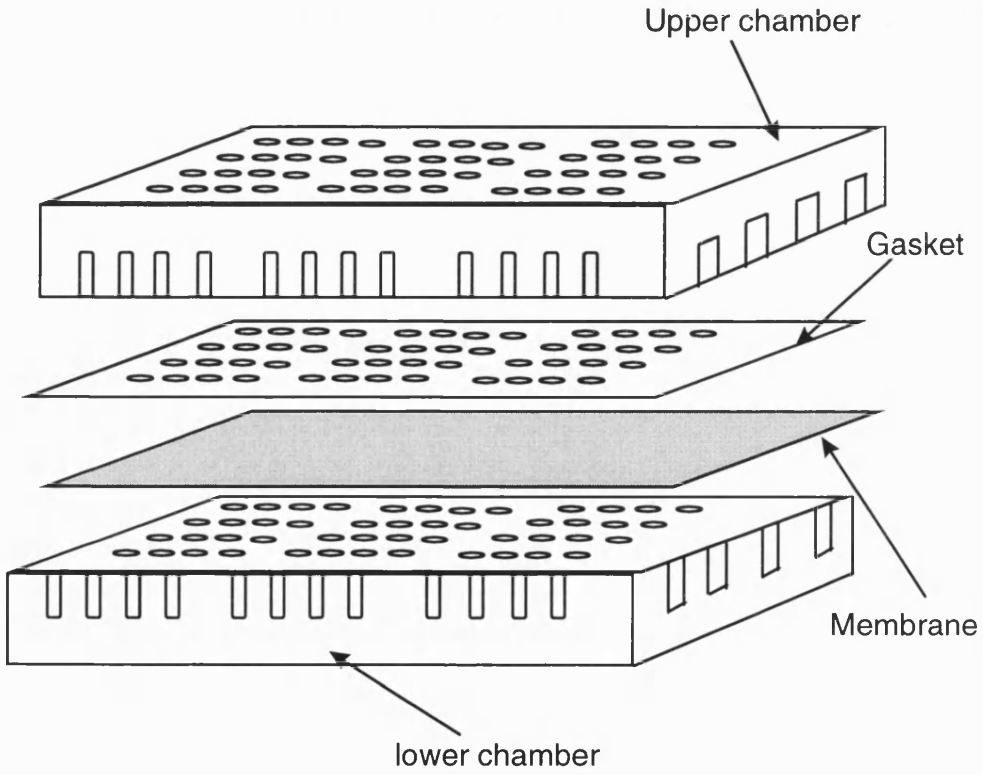


Figure 2.5 - Diagram of the Boyden Chamber set-up.
The agonist was placed in the lower chamber and the membrane and gasket were placed ontop. The upper chamber was secured in place and then after a 10 minute incubation period $7x10^4$-$1.6x10^5$ cells were placed in the upper chamber. Following incubation for 90-120 minutes the filter was washed, stained and counted as given in section 2.2.11.

thoroughly in PBS and the cells fixed in methanol and stained with Diff-Quick stain. The number of cells per 5 high power fields of view were counted.

2.2.12 Adhesion molecule expression

Human venous blood from healthy individuals was collected into 3.8% sodium citrate at 1ml per 9ml of blood. 100µl aliquots were stimulated with MCP-1 (1.25-60nM) for 30 minutes at 37°C. For the last 10 minutes the samples were incubated with 10µl FITC-labelled antibodies against CD11b, CD11c, VLA-4, IgG control and CD14 at room temperature and then washed twice in PBS containing 2% FCS and 0.05% azide (FACS buffer). The red blood cells were subsequently lysed using a red blood cell lysing solution ('Immunolyse') from Coulter. Briefly, 1ml of 1:25 diluted working solution of the lysis buffer was added and the samples vortexed vigorously. Then, between 30 seconds to 2 minutes later 250µl of fixative was added. Again, the samples were vortexed vigorously and 2ml of FACS buffer was added. The cells were pelleted by centrifugation and washed twice with FACS buffer. The samples were resuspended in 500µl of FACS buffer and analysed on a Becton Dickinson FACScan, collecting 10 000 events per sample, the monocyte population being selected from both the scatter pattern and the CD14 positive staining.

2.2.13 Measurement of superoxide release from monocytes.

There are a number of ways in which the generation of superoxide from cells can be measured (Jones & Hancock, 1994). The two methods utilised in this study were the oxidation of scopoletin to a non-fluorescent product by H_2O_2, which is the product of the spontaneous breakdown of superoxide and the direct reduction of ferricytochrome C by superoxide.

2.2.13.1 Scopoletin assay (De La Harpe & Nathan, 1985)

Monocytes were purified as given in section 2.2.1.3 and resuspended in HBSS containing 0.1% BSA and 1.5mM Ca^{2+} and Mg^{2+} at a concentration of $2x10^6$ cells/ml. For the standard curve, 10µl of H_2O_2 standards (0.5-6nmol), diluted in H_2O, were added followed by 100µl of HBSS alone and finally 10µl of the reaction mixture (appendix 6). For the samples, 10µl of reaction mixture was added followed by the addition of 100µl of cell suspension. A zero reading of the plate was taken immediately prior to the reaction being started by the addition of the agonist or vehicle. The plate was read on a Titretek Fluoroskan II fluorescent plate reader at an excitation wavelength of 355nm and an emission wavelength of 460nm at 10 minute intervals for up to 120 minutes. The results were then calculated from the standard curve using equation 3. A typical standard curve is shown in figure 2.6.

Equation 3

The equation of the line for the standard curve is:-

$y = y_o + A\, e^{-\frac{(x - xo)}{t}}$ where y_o, A and t are constants obtained from the graph.

Therefore, solving for x:-

$$y - y_o = A\, e^{-\frac{x}{t}}$$

$$\frac{y-y_o}{A} = e^{-\frac{x}{t}}$$

$$-\left[\ln\left[\frac{y-y_o}{A}\right]\right] t = x$$

2.2.13.2 Ferricytochrome C assay (Pick & Mizel, 1981)

The monocytes were purified in a slightly different manner for this assay. The PBMC's obtained from the Lymphoprep separation given in section 2.2.1.3 were plated out in a 96 well plate at $1x10^6$ cells/well.

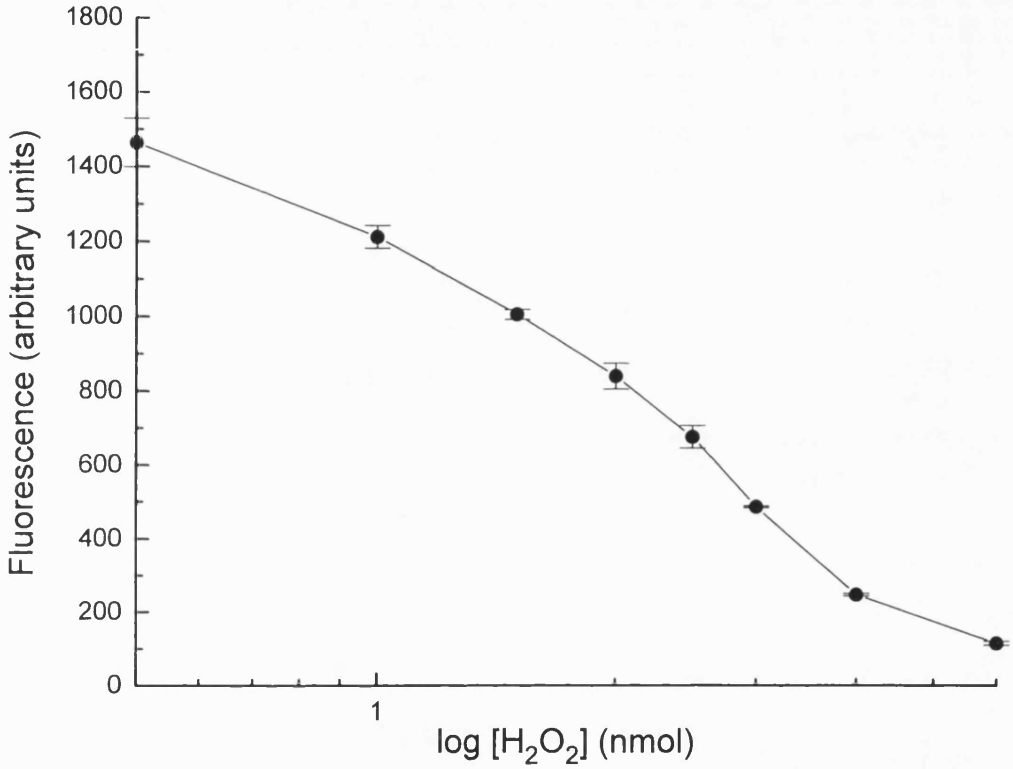


Figure 2.6 - A typical standard curve for the measurement of hydrogen peroxide generation using scopoletin. Data is mean +/- s.d. of triplicate samples.

After $1^1/_2$ hours adherence at 37^0C the non adherent cells were washed off and the adherent cells covered with 50µl HBSS with 1mM Ca^{2+}/Mg^{2+} and 0.1% BSA. 50µl of 320µM ferricytochrome C solution was added to each well either with or without 300U/ml SOD. The reaction was started by the addition of 10µl agonist or vehicle (made up in 160µM ferricytochrome C solution) and the plate was read at 550nm on a Dynatech MR5000 plate reader at 10 minute intervals for up to 120 minutes.

The background values of the wells containing no agonist plus SOD were subtracted from the values of the sample wells and the nmole concentration of superoxide present per well was calculated using the extinction coefficient of reduced ferricytochrome C, $21x10^3M^{-1}cm^{-1}$ (see equation 4). A protein assay (section 2.2.13.2.1) was then carried out to determine the amount of protein per well and if the protein concentrations were significantly variable then the values adjusted to nmoles superoxide / mg protein.

Equation 4

Extinction coefficient of reduced cytochrome C (O.D.) = $21 \times 10^3 M^{-1} cm^{-1}$

However, the light path was not 1cm but 0.3cm.

Therefore, extinction coefficient (O.D.) = $6.3 \times 10^3 M^{-1} 0.3cm^{-1}$

Thus, in 100µl well volumes, nmoles of superoxide ≡ nmoles of reduced cytochrome C

$$= \frac{\text{O.D.} \times 100}{6.3}$$

2.2.13.2.1 Protein assay

The cells were digested for 18 hours with 100µl 1M NaOH at 4^oC. 50µl of each sample was then diluted by 1:2 with water to reduce the effects of the NaOH on the assay. The BSA standards (3.125-100µg/ml) were also made up in 0.5M NaOH. 20µl Biorad reagent was added to each well and the solutions were thoroughly mixed.

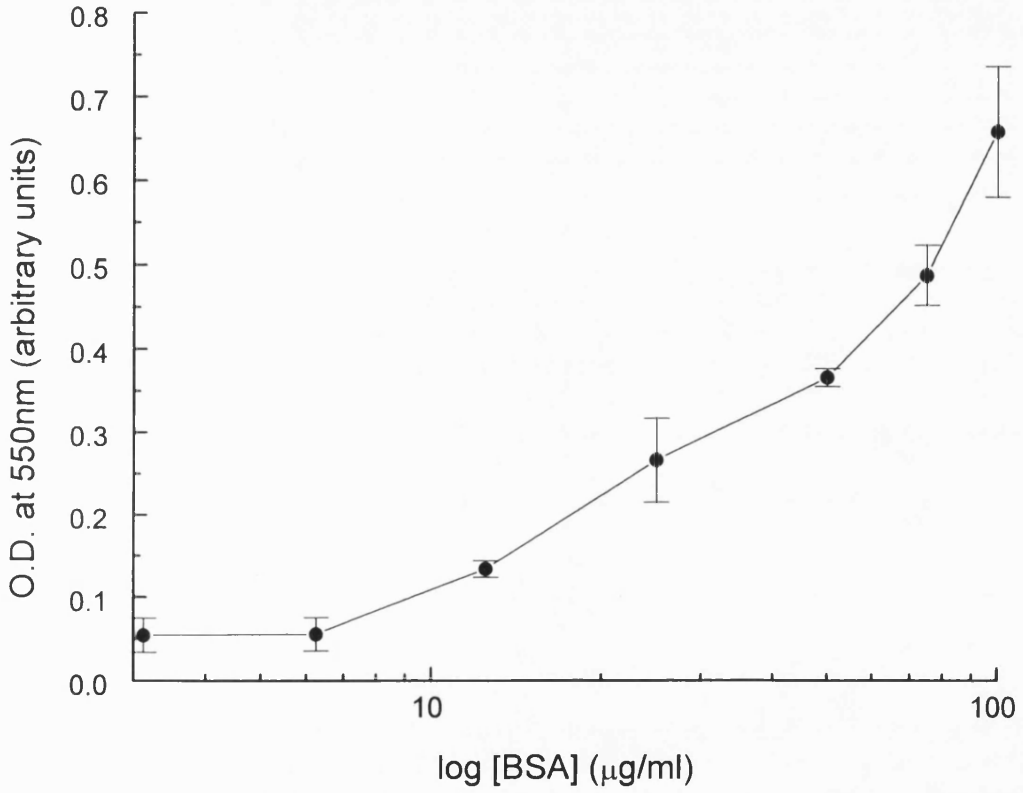


Figure 2.7 - A typical standard curve for the measurement of protein content in the samples used in the ferricytochrome C assay. Data is mean +/- s.d. of triplicate samples.

The plate was read at 595nm on a Dynatech MR5000 plate reader. The protein concentrations were calculated by linear regression from the standard curve (see figure 2.7 for an example) and then if significantly variable, the superoxide results were adjusted.

2.2.14 CD23 upregulation on human monocytes

The PBMC's were purified as given in section 2.2.1.3. These were put into Petri dishes at between $2x10^6$-$5x10^6$ cells/ml and incubated with the agonists for 44 hours. After incubation, the cells were washed and aliquoted into separate tubes. The samples were incubated with 10µl of anti-CD14-, -IgG_1- , -IgG_{2A}- or -CD23-FITC antibody for 10 minutes at room temperature in FACS buffer (see section 2.2.12). The cells were subsequently washed three times in PBS and resuspended in 500µl of FACS buffer. The samples were analysed using a Beckton Dickinson FACScan, collecting 10 000 events per sample, selecting the monocyte population by scatter properties and CD14 positive staining.

2.2.15 Statistical analysis

Unless otherwise stated in the text, where appropriate, statistical analysis was performed on the raw data from 'n' experiments using 2-way Analysis of Variance (ANOVA) and Dunnet's T-test. $P<0.05$, $P<0.01$ and $P<0.001$ are represented by *,** and *** respectively.

SECTION 3 : MCP-1 BINDING STUDIES

3.1 [^{125}I]-MCP-1 binding

The initiation of any MCP-1-induced responses must be preceded by the binding of MCP-1 to its receptor. To study this interaction initially, the classical radioligand binding study was employed. The optimal concentration of [^{125}I]-MCP-1 was determined by Dr. M. Needham at Zeneca, U.K. by incubating cells with increasing concentrations of [^{125}I]-MCP-1 (~0.01-10nM). The time taken for equilibrium to be reached was also determined by incubating cells with [^{125}I]-MCP-1 for various times (~10-120 minutes). The optimal [^{125}I]-MCP-1 concentration was observed to be 100pM with equilibrium being reached between 45-90minutes (Dr. M. Needham, Zeneca, U.K., personal communication).

3.1.1 THP-1 cells

[^{125}I]-MCP-1 was observed to bind to THP-1 cells. Figure 3.1a shows the competition curve obtained for THP-1 cells with increasing concentrations of competing MCP-1 (0.01-30nM). The Scatchard analysis of this saturation binding curve is shown in figure 3.1b. The Scatchard plot was a straight line from which the Kd was determined as 4.71 ± 0.91nM (n=4). These results together indicate the presence of a single group of high affinity MCP-1 receptors. The number of these receptors per cell was calculated from the B_{MAX} to be 19 979 ± 3 508 receptors per cell (n=4).

3.1.2 CC CKR 2A and 2B transfected cells

There was a complete absence of [^{125}I]-MCP-1 binding to the CC CKR 2A transfected cells. This is demonstrated in figure 3.2a, in which the radioactivity bound to the CC CKR 2A cells is plotted alongside the radioactivity bound to the CC CKR 2B cells. The maximum binding detected in the CC CKR 2A cells was only 118.6 ± 24.5 cpm (n=4),

compared to the maximum binding detected in the CC CKR 2B transfected cells which was 1 256.7 ± 41.7 cpm (n=4).

The [^{125}I]-MCP-1 binding detected in the CC CKR 2B cells was very similar to that observed with THP-1 cells. Figure 3.2b shows the competition curve with increasing concentrations of unlabelled MCP-1. Scatchard analysis of this data also demonstrated a straight line with a Kd of 2.69 ± 0.28nM (figure 3.2c). The number of receptors per cell was calculated to be 22 618 ± 7 593 (n=4).

3.2 Biotinylated MCP-1 binding

Due to the lack of [^{125}I]-MCP-1 binding detected to the CC CKR 2A transfected cells, a relatively new technique was employed. This utilised biotinylated MCP-1 which bound to the receptor and this was detected by using an avidin-FITC conjugate, allowing MCP-1 binding to individual cells to be analysed using the FACS.

3.2.1 THP-1 cells

Figure 3.3a shows the changes in fluorescence detected in the presence or absence of biotinylated MCP-1 as well as the effects of an anti-MCP-1 antibody. In the presence of biotinylated MCP-1 there was an increase in the mean fluorescence intensity above that of control, demonstrating MCP-1 binding (mean fluorescence intensity for the control=11.79 ± 0.91, mean fluorescence intensity for biotinylated MCP-1=29.9 ± 3.1; $P<0.05$). This MCP-1 binding was completely abrogated in the presence of anti-MCP-1 antibody demonstrating that the binding was specific. A similar inhibition was also observed with the non-biotinylated MCP-1 (figure 3.3b) confirming the specificity of the MCP-1 binding.

3.2.2 CC CKR 2A and 2B transfected cells

Figure 3.4a shows the increase in fluorescence detected with the CC CKR 2A cells. In contrast to the results observed with the radioligand binding, MCP-1 was demonstrated to bind to the CC CKR 2A transfected cells (control=18.93 ± 1.42, biotinylated MCP-1=36.0 ± 4.0; $P<0.05$). The complete inhibition of this binding by both the anti-MCP-1 antibody and the non-biotinylated MCP-1 established that this binding was specific (figure 3.4a and b).

The CC CKR 2B cells also demonstrated specific MCP-1 binding as shown in figures 3.4c and d (mean fluorescence intensities, control=25.22 ± 3.2, biotinylated MCP-1=57.65 ± 12.4; $P<0.05$). The increase in fluorescence was completely inhibited by both the anti-MCP-1 antibody and non-biotinylated MCP-1. One noticeable difference observed between the CC CKR 2A and 2B transfected cells was the pattern of the fluorescence increases. The CC CKR 2B cells demonstrated similar results to those observed in the THP-1 cells. A single peak was observed to shift to the right in the presence of biotinylated MCP-1. In contrast, the CC CKR 2A transfected cells demonstrated two populations of cells, indicated by the split peak in the presence of biotinylated MCP-1. The first peak was not noticeably different from the control but the second peak was much higher than that of the control.

There was no increase in MCP-1 binding above that of control in the untransfected HEK 293 cells (figure 3.5).

3.3 Summary

1. MCP-1 bound to a single group of high affinity receptors on THP-1 cells with a Kd of 4.71 ± 0.91nM (n=4). The number of these high affinity receptors per cell was determined from the Scatchard plot as 19 979 ± 3 508 (n=4). This binding was

confirmed by the significant increase in fluorescence observed in these cells in the presence of biotinylated MCP-1. The specificity of this binding was demonstrated by the reduction in the fluorescence by anti-MCP-1 antibody and non-biotinylated MCP-1.

2. No detectable [^{125}I]-MCP-1 binding was observed to the CC CKR 2A transfected cells. In contrast, biotinylated MCP-1 was observed to specifically bind to a population of these cells. The specificity was again determined by the reduction in fluorescence by both anti-MCP-1 antibody and non-biotinylated MCP-1. Two populations of cells, one showing MCP-1 binding and the other not, were demonstrated by the two peaks observed in the FACS data in the presence of biotinylated MCP-1.
3. CC CKR 2B cells demonstrated similar binding characteristics to those observed in THP-1 cells. The Kd and the number of receptors per cell were calculated from the Scatchard plot as 2.69 ± 0.28nM and 22 618 ± 7 593 respectively. Specific binding was confirmed by biotinylated MCP-1 binding and its inhibition by anti-MCP-1 antibody and non-biotinylated MCP-1.

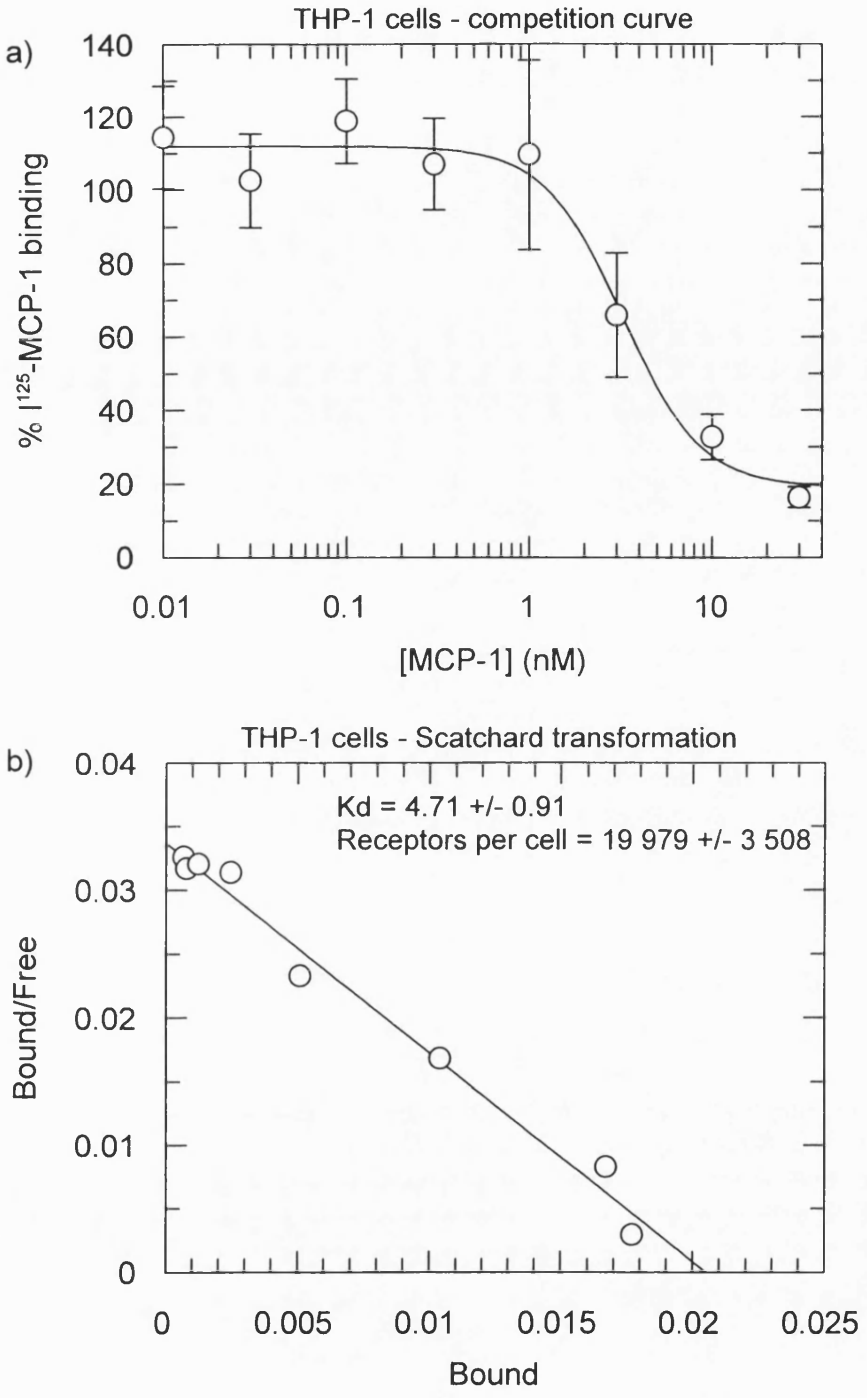


Figure 3.1 - [^{125}I]-MCP-1 binding to THP-1 cells.
a) 1x10^6 THP-1 cells were incubated with 100pM [^{125}I]-MCP-1 and increasing concentrations of competing MCP-1 as given in section 2.2.2. The results are shown as mean +/- s.e.m. (n=4).
b) Scatchard analysis of the competition data. This data is representative of four separate experiments. Analysis of the data was performed using the Grafit binding program.

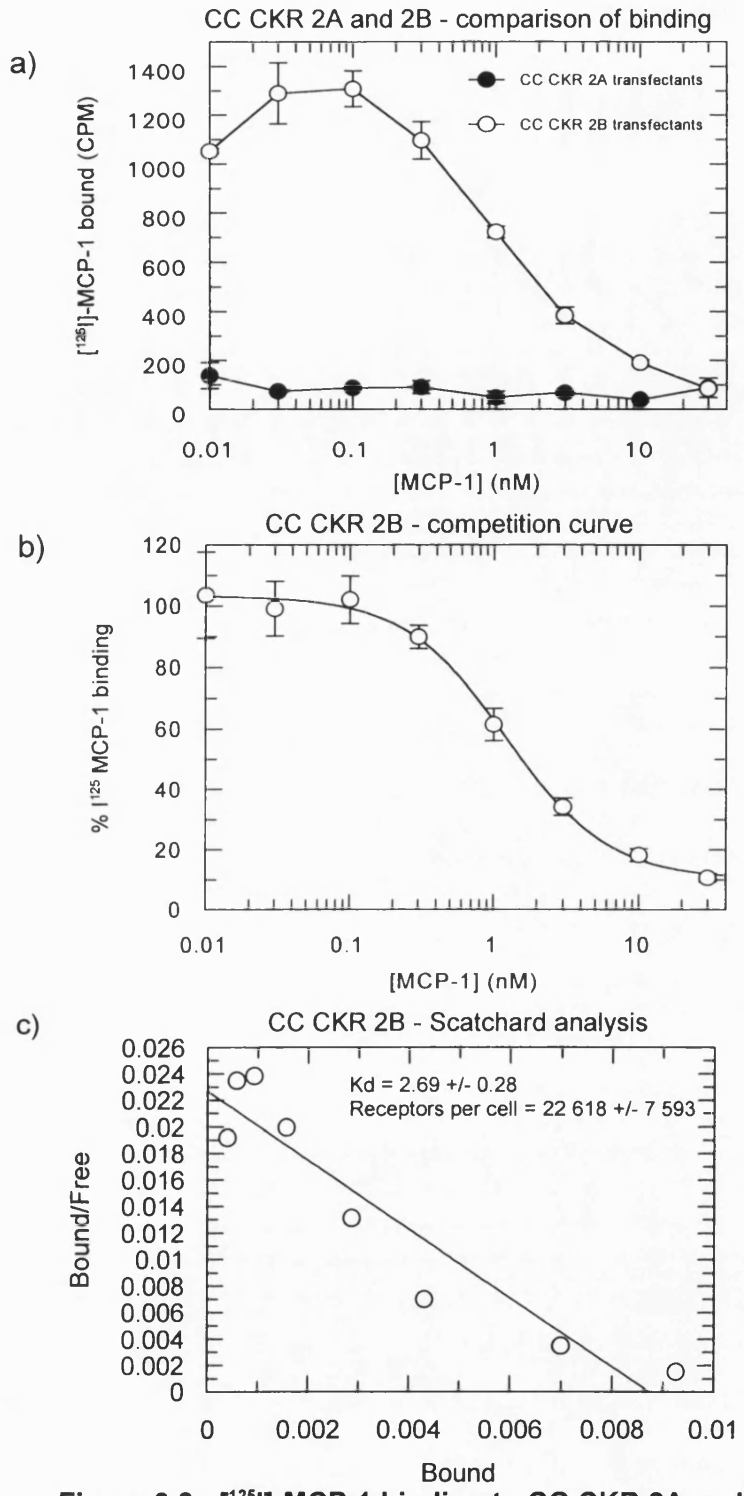


Figure 3.2 - [^{126}I]-MCP-1 binding to CC CKR 2A and 2B transfected cells. a) 5x10^5 CC CKR 2A or 2B cells were incubated with 100pM [^{125}I]-MCP-1 and increasing concentrations of competing MCP-1 as given in section 2.2.2. The results are shown as mean CPM +/- s.d. and are representative of four separate experiments. b) Competition curve in the CC KR 2B cells. The results are expressed as percentage binding and are shown as mean +/- s.e.m. (n=4). c) Scatchard analysis of the competition data in the CC CKR 2B cells. This data is representative of four separate experiments. Analysis of the data was performed using the Grafit binding program.

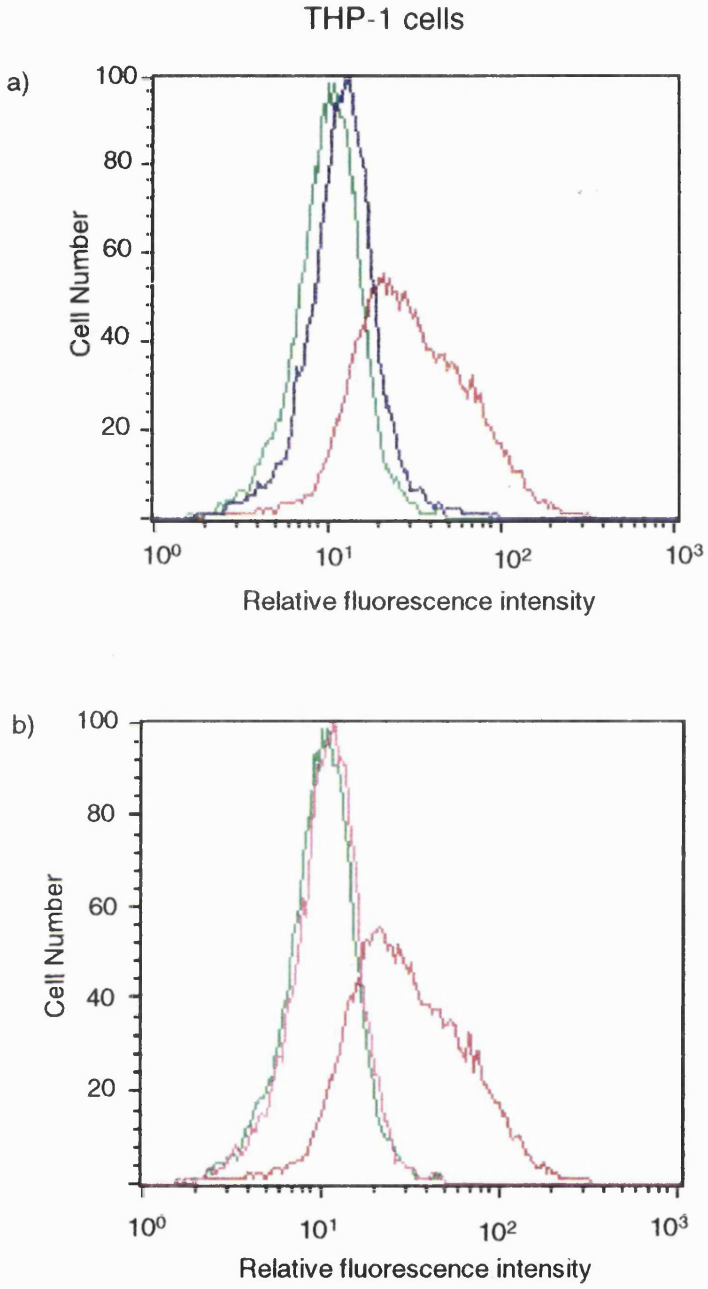


Figure 3.3 - Binding of biotinylated MCP-1 to THP-1 cells. $1x10^5$ cells were incubated with biotinylated MCP-1 for 60 minutes followed by an avidin-FITC conjugate for the next 30 minutes as given in section 2.2.3. The samples were analysed by FACS. The data is representative of three separate experiments and shows the increase in fluorescence in the presence of biotinylated MCP-1 (—), biotinylated MCP-1 with anti-MCP-1 antibody (—), biotinylated MCP-1 with non-biotinylated MCP-1 (—) or control (—).

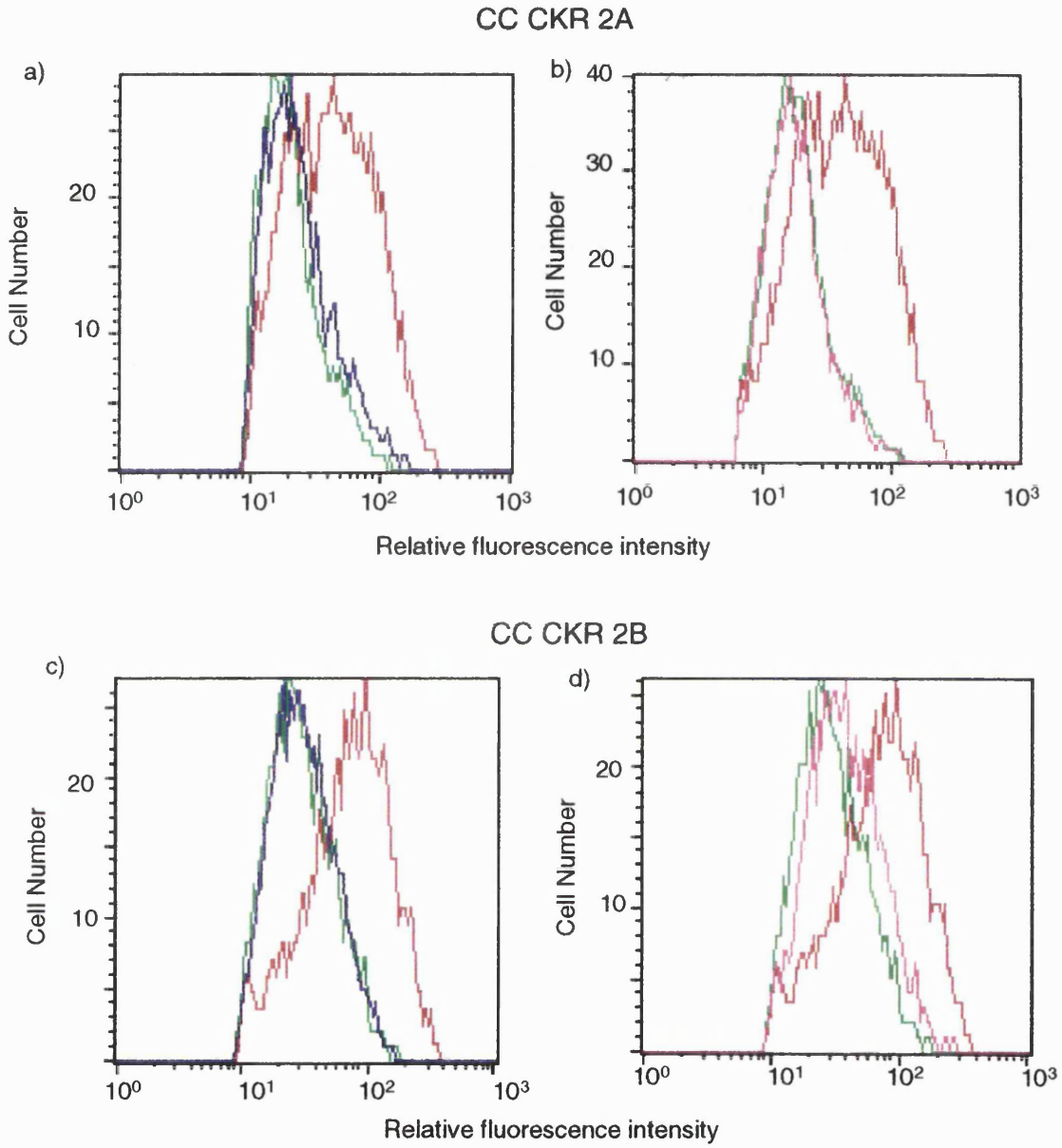


Figure 3.4 - Binding of biotinylated MCP-1 to a) and b) CC CKR 2A and c) and d) CC CKR 2B transfected cells. $1x10^5$ cells were incubated with biotinylated MCP-1 for 60 minutes followed by an avidin-FITC conjugate for the next 30 minutes as given in section 2.2.3. The samples were analysed by FACS. The data is representative of three separate experiments and shows the increase in fluorescence in the presence of biotinylated MCP-1 (—), biotinylated MCP-1 with anti-MCP-1 antibody (—), biotinylated MCP-1 with non-biotinylated MCP-1(—) or control (—).

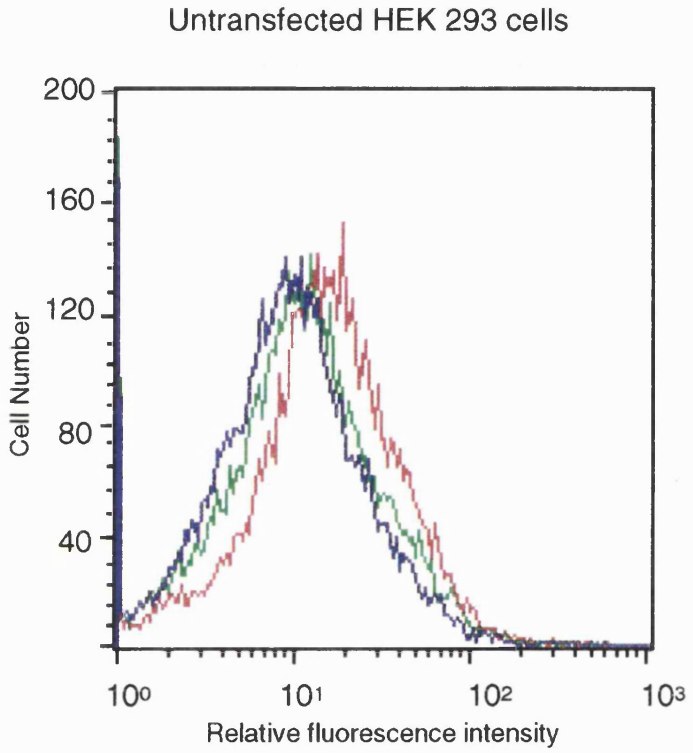


Figure 3.5 - Binding of biotinylated MCP-1 to untransfected HEK 293 cells. 1x10^5 cells were incubated with biotinylated MCP-1 for 60 minutes followed by an avidin-FITC conjugate for the next 30 minutes as given in section 2.2.3. The samples were analysed by FACS. The data is representative of three separate experiments and shows the increase in fluorescence in the presence of biotinylated MCP-1 (—), biotinylated MCP-1 with anti-MCP-1 antibody (——) or control (—).

SECTION 4 : CHARACTERISATION OF THE MCP-1-INDUCED RISE IN $[Ca^{2+}]_i$

4.1 Measurement of $[Ca^{2+}]_i$ in THP-1 cells

4.1.1 Elevation of $[Ca^{2+}]_i$ by MCP-1

One of the most well documented chemokine responses is the increase in $[Ca^{2+}]_i$ (Oppenheim *et al.* 1991). Figure 4.1a demonstrates the increase in $[Ca^{2+}]_i$ observed in THP-1 cells following MCP-1 stimulation. This rise was dose-dependent and transient in nature, reaching a maximum by 10 seconds and returning to almost basal levels by 60 seconds. Figure 4.1b shows the dose-response curve for the MCP-1-induced increase in $[Ca^{2+}]_i$. The EC_{50} for MCP-1 was 2.13 ± 0.2nM (n=6) and the maximal change in $[Ca^{2+}]_i$ following 12.5nM MCP-1 stimulation was 230.14 ± 45.66nM (n=6).

4.1.2 Desensitisation of the MCP-1-induced calcium response

The calcium response detected following MCP-1 stimulation can be used as a method for identifying the receptors expressed by THP-1 cells. Repeated stimulation of a chemokine receptor with the same chemokine, or another chemokine acting at the same receptor, leads to desensitisation of the second response (Sozzani *et al.* 1993)(Dahinden *et al.* 1994)(Combadiere *et al.* 1995). This is a well reported chemokine receptor characteristic and provides a useful method for identifying the receptors present on the cells by sequential stimulation with a selection of chemokines. Figures 4.2, 4.3 and table 4.1 summarise the results seen following repeated stimulation of THP-1 cells by a variety of different chemokines. Stimulation with 12.5nM MCP-1 almost completely abrogated the calcium response to a second stimulation with 12.5nM MCP-1.

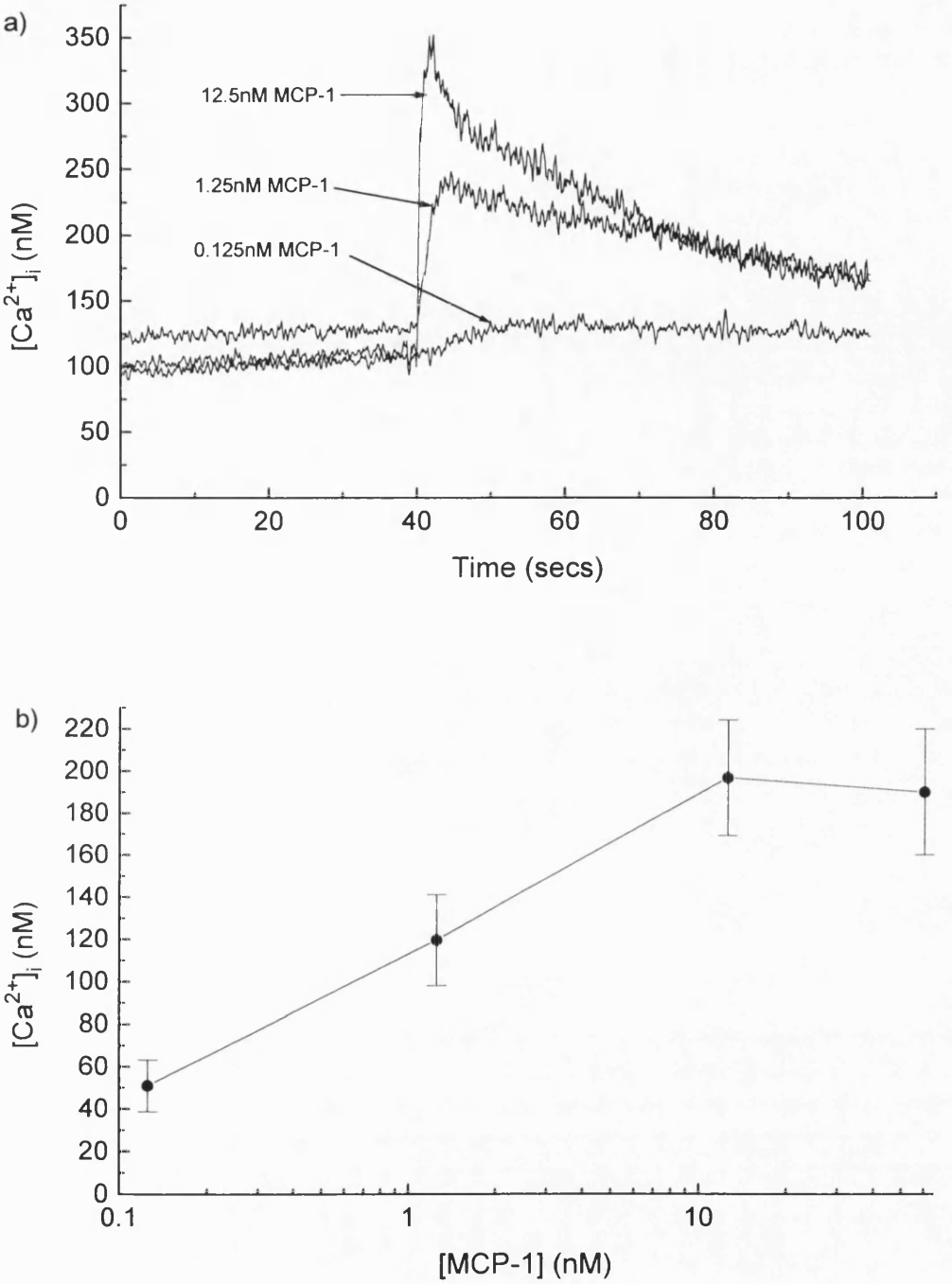


Figure 4.1 - Dose-dependent effects of MCP-1 on the $[Ca^{2+}]_i$ response in THP-1 cells. 10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The increases in $[Ca^{2+}]_i$ in response to three concentrations of MCP-1. The data is representative of at least five separate experiments. b) The dose-response curve for MCP-1-induced $[Ca^{2+}]_i$ changes (mean data +/- s.e.m., n=5)

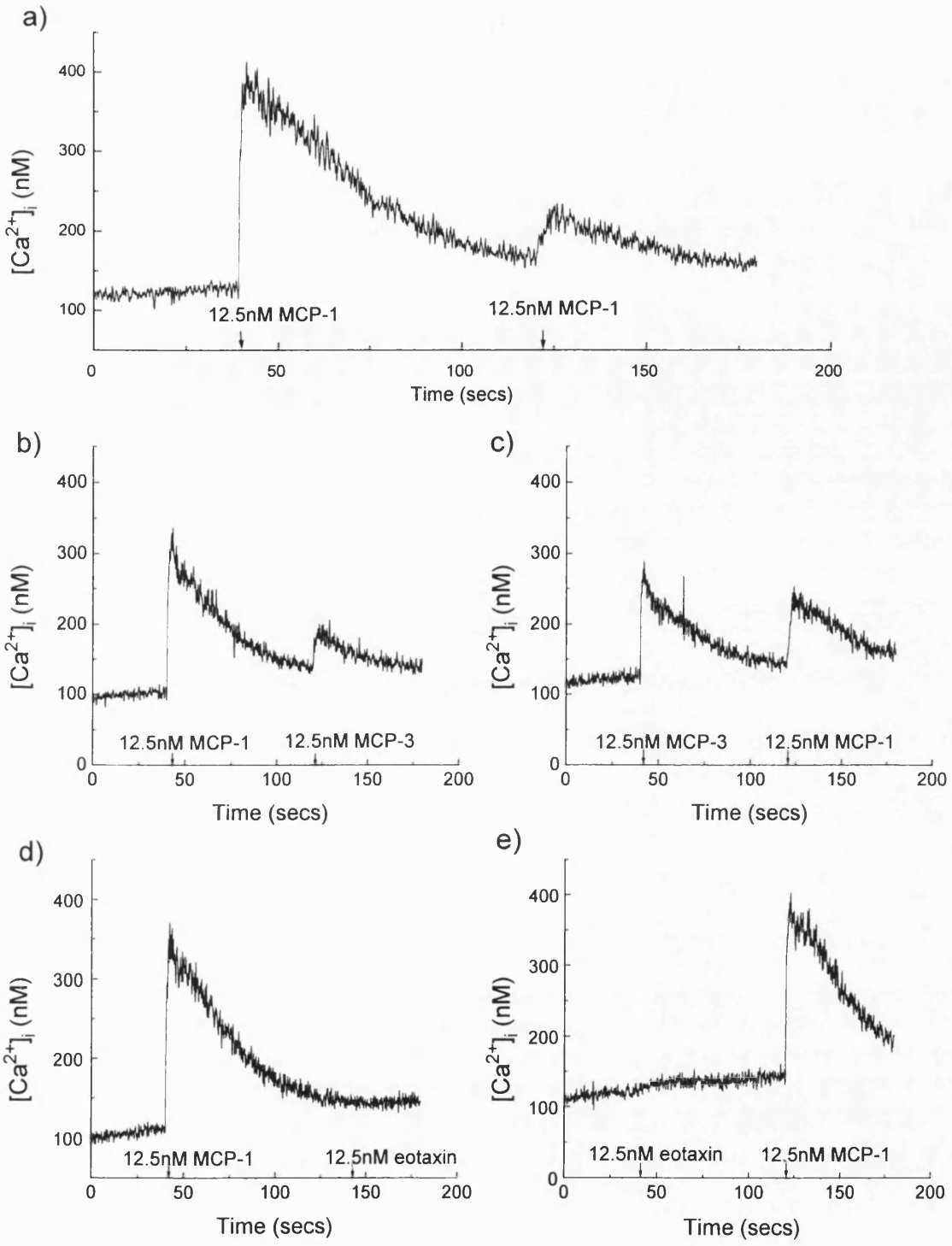


Figure 4.2 - Desensitisation effects of repeated chemokine stimulation.
10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. Stimulation with the first chemokine was at 40 seconds followed by the second chemokine 80 seconds later. All chemokines were used at a concentration of 12.5nM. a) shows the effects of repeated doses of MCP-1. b) and c) show the effects of MCP-1 and MCP-3 and d) and e) show the effects of MCP-1 and eotaxin. The traces are representative of three separate experiments.

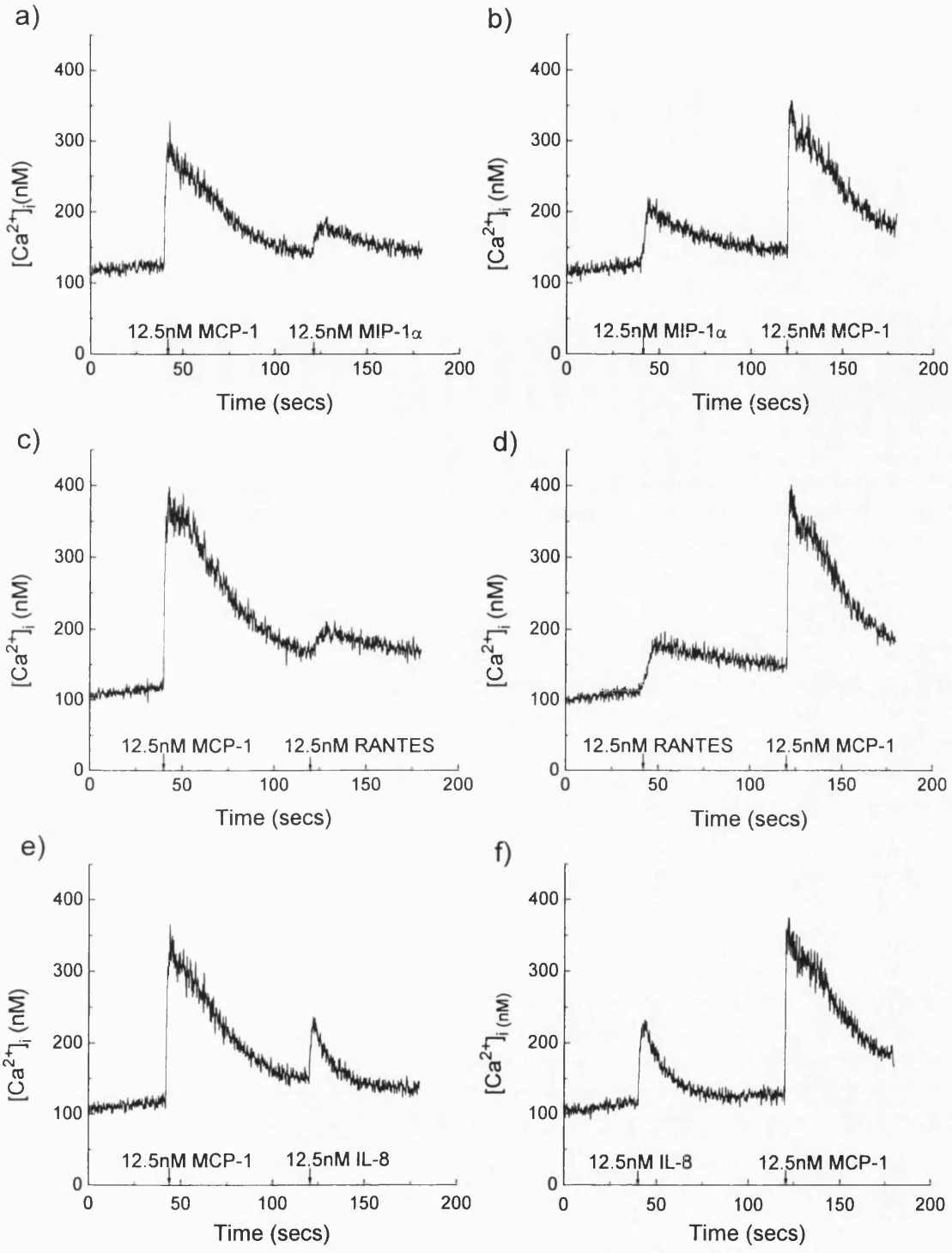


Figure 4.3 - Desensitisation effects of repeated chemokine stimulation.
10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. Stimulation with the first chemokine was at 40 seconds and with the second chemokine stimulation 80 seconds later. All the chemokines were used at a concentration of 12.5nM. a) and b) show the effects of MCP-1 and MIP-1α, c) and d) show the effects of MCP-1 and RANTES and e) and f) show the effects of MCP-1 and IL-8. The traces are representative of three separate experiments.

MCP-1 also significantly reduced the calcium responses to sequential MCP-3, RANTES, MIP-1α and IL-8 stimulation. However, only MCP-3 could significantly inhibit the subsequent MCP-1-induced calcium response.

4.1.3 Identification of the source of calcium responsible for the MCP-1-induced $[Ca^{2+}]_i$ elevation

4.1.3.1 Role of calcium influx in the MCP-1-induced rise in $[Ca^{2+}]_i$

In order to be able to study the role of both calcium influx and calcium mobilisation in the MCP-1-induced calcium response, a positive control for both of these calcium sources was required. ATP has already been reported to induced a biphasic calcium response, involving both calcium influx and calcium mobilisation, in macrophages (Greenberg *et al.* 1988). When applied to THP-1 cells, ATP did induce a dose-dependent increase in $[Ca^{2+}]_i$ (figure 4.4a and b). The response was very different to that observed following MCP-1 stimulation. Although the increase was very rapid, it remained maximally elevated for more than 60 seconds.

To be able to identify the source of the $[Ca^{2+}]_i$ rise in response to MCP-1 and compare it to the ATP response, a number of inhibitors were utilised. These included the calcium chelator EGTA (Hagiwara & Nakajima, 1966), the calcium channel blocker Ni^{2+} (Hagiwara & Takahashi, 1967) and the calcium influx inhibitor econazole (Alvarez *et al.* 1991).

Figures 4.5a and b show the effects of EGTA on the MCP-1-induced increase in $[Ca^{2+}]_i$. EGTA had no obvious effects on the peak height of the response, although the $T_{1/2}$ was reduced. The $T_{1/2}$ is defined as the time taken for the response to fall to half of the maximum height. In contrast to the effects of EGTA, addition of 1mM Ni^{2+} prior to MCP-1 addition inhibited the MCP-1-induced calcium response by

Table 4.1 - Effects of repeated chemokine addition on the $[Ca^{2+}]_i$ in THP-1 cells. The cells were loaded as given in section 2.2.4. Addition of the first agonist was at 40 seconds followed by the addition of the second agonist at 120 seconds. a) This shows the effects of initial stimulation with MCP-1 on the subsequent responses to various chemokines and b) shows the effects of the various chemokines on the subsequent MCP-1 response. All agonists were added at a concentration of 12.5nM. The results are expressed as a percentage of the control response generated by the second agonist when the first agonist was only HBSS (mean ± s.e.m, n=3).

a)

Agonist (12.5nM)		Percentage of control response
First	**Second**	
MCP-1	MCP-1	12.47 ± 2.7 **
MCP-1	MCP-3	22.1 ± 4.6 **
MCP-1	RANTES	39.3 ± 7.4 **
MCP-1	MIP-1α	39.5 ± 4.8 **
MCP-1	Eotaxin	No response
MCP-1	IL-8	52.05 ± 14.73 **

b)

Agonist (12.5nM)		Percentage of control response
First	**Second**	
MCP-1	MCP-1	12.47 ± 2.7 **
MCP-3	MCP-1	48.3 ± 4.4 **
RANTES	MCP-1	86.7 ± 6.9
MIP-1α	MCP-1	95.5 ± 4.5
Eotaxin	MCP-1	100
IL-8	MCP-1	100

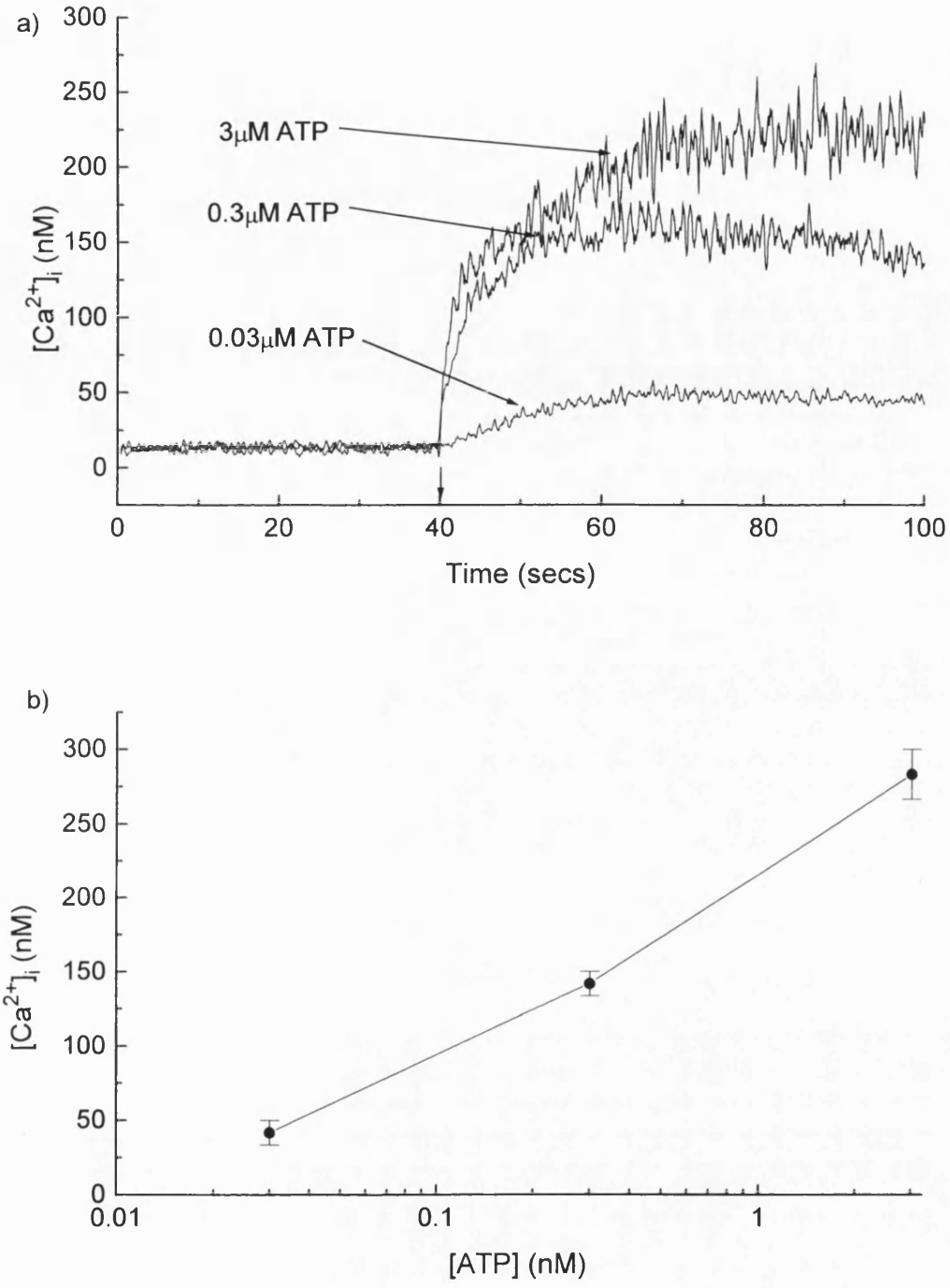


Figure 4.4 - Dose-dependent effects of ATP on $[Ca^{2+}]_i$ in THP-1 cells.
10^6-10^7 THP-1 cells were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The increases in $[Ca^{2+}]_i$ in response to three concentrations of ATP. The data is representative of at least five separate experiments. b) The dose-response curve for ATP-induced $[Ca^{2+}]_i$ changes (mean +/- s.e.m., n=5)

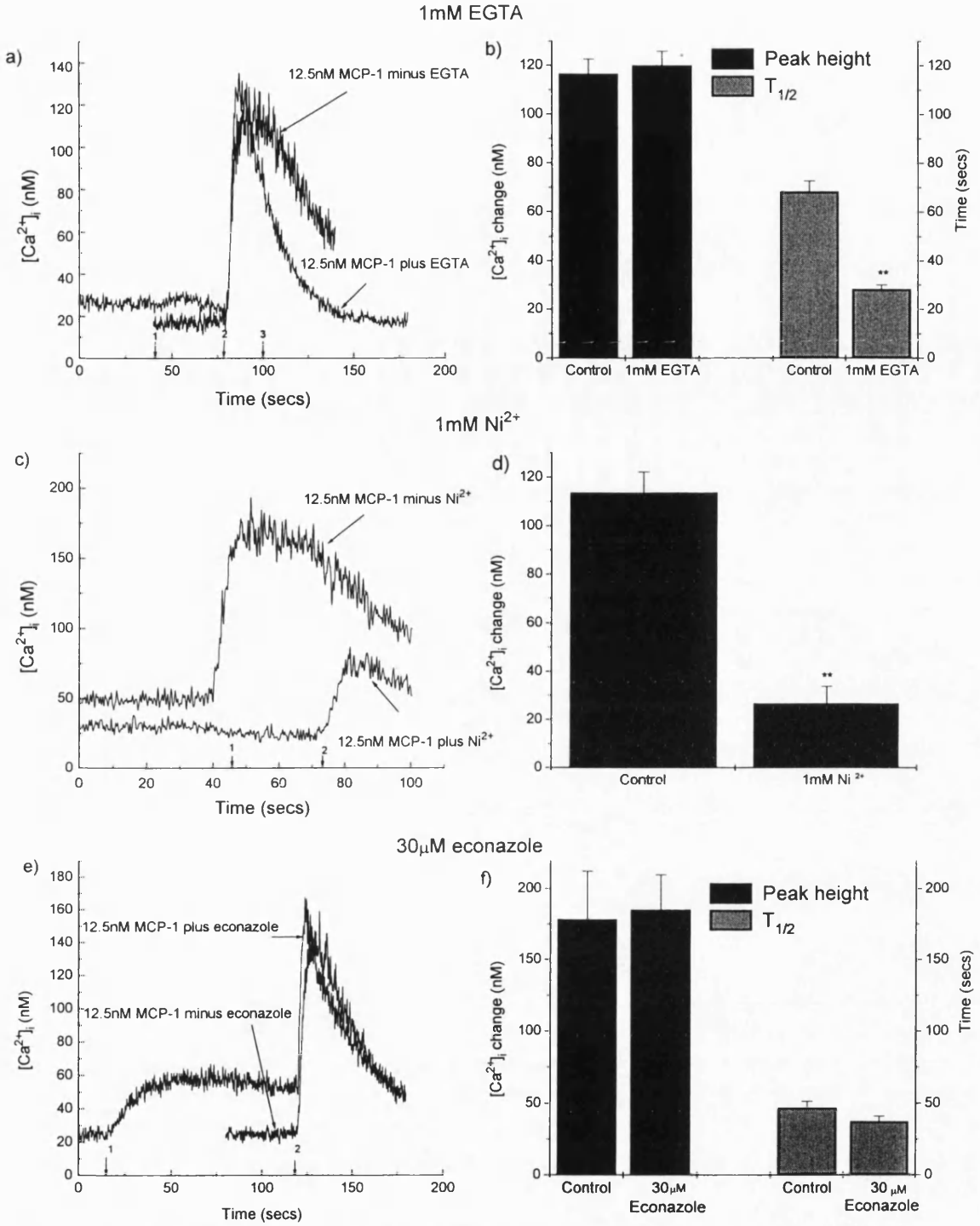


Figure 4.5 - Effects of calcium influx inhibitors on the MCP-1-induced increase in $[Ca^{2+}]_i$. 10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) 1mM EGTA was added at 40 seconds (1) followed by 12.5nM MCP-1 (2) 40 seconds later. 1mM Ca^{2+} was readded at 100seconds (3). b) Graphical representation of the effects of EGTA on the peak height and the $T_{1/2}$ of the MCP-1 response. c)1mM Ni^{2+} was added to the cells at 40 seconds and the MCP-1-induced calcium response in the presence or absence of Ni^{2+} was monitored. d) Graphical representation of the effects of Ni^{2+} on the MCP-1-induced calcium response e) 30μM econazole was added at 10 seconds (1) and then 12.5nM MCP-1 was added after a 2 minute incubation period (2). f) Graphical representation of the effects of econazole on the peak height and the $T_{1/2}$ of the MCP-1 response. The traces are all representative of five separate experiments and the graphs are all mean +/- s.e.m., n=5.

68.9 ± 11.7% (n=5) (figure 4.5c and d). Figures 4.5e and f show the effects of econazole on the MCP-1-induced response. A 2 minute pre-treatment of the cells with 30μM econazole had no significant effect on either the peak height or the $T_{1/2}$ of the MCP-1-induced increase in $[Ca^{2+}]_i$.

These results are in stark contrast to the results obtained with ATP. Figures 4.6a and b demonstrate that EGTA almost completely abrogated the prolonged phase of the ATP-induced calcium response. Inhibition of the response 40 seconds after ATP addition was 60.6 ± 1.2% (n=5) in the presence of EGTA. The readdition of 1mM Ca^{2+} returned the response to a level similar to that seen in the absence of EGTA. Similar results were also observed in the presence of Ni^{2+} and econazole. Both of these compounds greatly reduced the prolonged phase of the response (figure 4.6 c, d, e and f) (53.75 ± 13.9% (n=5) and 67.45 ± 8.99% (n=5) inhibition of the response 40 seconds after ATP administration respectively). None of these inhibitors had any effect on the initial peak of the ATP response.

Calcium influx can be further demonstrated by the use of Mn^{2+} as a surrogate Ca^{2+} ion. When Mn^{2+} enters the cell it binds to fura-2 and quenches the fluorescence signal. By monitoring at the isobestic point of fura-2, influx of Mn^{2+} can be directly monitored.

Figures 4.7a and b show the influx of Mn^{2+} in response to various concentrations of MCP-1 compared to buffer control and ionomycin as a positive control. There was no significant difference in the influx detected in response to any concentration of MCP-1 or buffer control. In contrast, figures 4.7c and d show the effects of increasing concentrations of ATP on the Mn^{2+} influx. ATP induced a significant dose-dependent influx of Mn^{2+}.

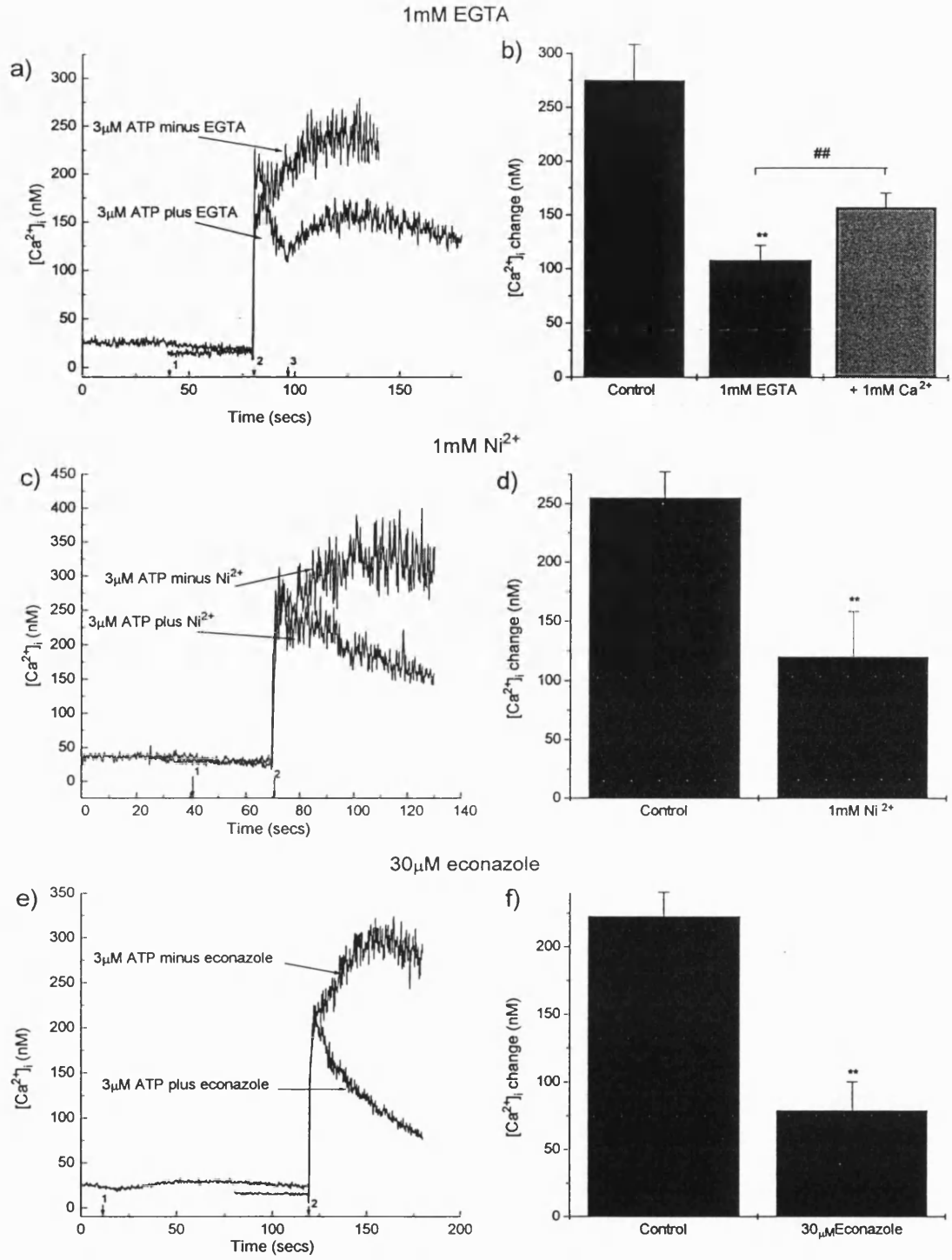


Figure 4.6 - Effects of calcium influx inhibitors on the ATP-induced increase in $[Ca^{2+}]_i$.

10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) 1mM EGTA was added at 40 seconds (1) followed by 3μM ATP (2) 40 seconds later. 1mM Ca^{2+} was readded (3) at 100 seconds. b) Graphical representation of the effects of EGTA The measurements were taken either 40 seconds after ATP addition for the control, 20 seconds after ATP and 40 seconds after Ca^{2+} readdition in the presence of EGTA. c) Either 3μM ATP alone (2) or 1mM Ni^{2+} (1) followed by ATP (2) 30 seconds later were added to the cells. d) Graphical representation of the effects of Ni^{2+}. The measurements were taken 40 seconds after ATP addition. e) 30μM econazole was added at 10 seconds (1) and 3μM ATP was added 2 minutes later (2). f) Graphical representation of the effects of econazole. The measurements were taken 40 seconds after ATP addition. The traces are all representative of five separate experiments. The graphs are all expressed as mean $[Ca^{2+}]_i$ change +/- s.e.m., n=5.

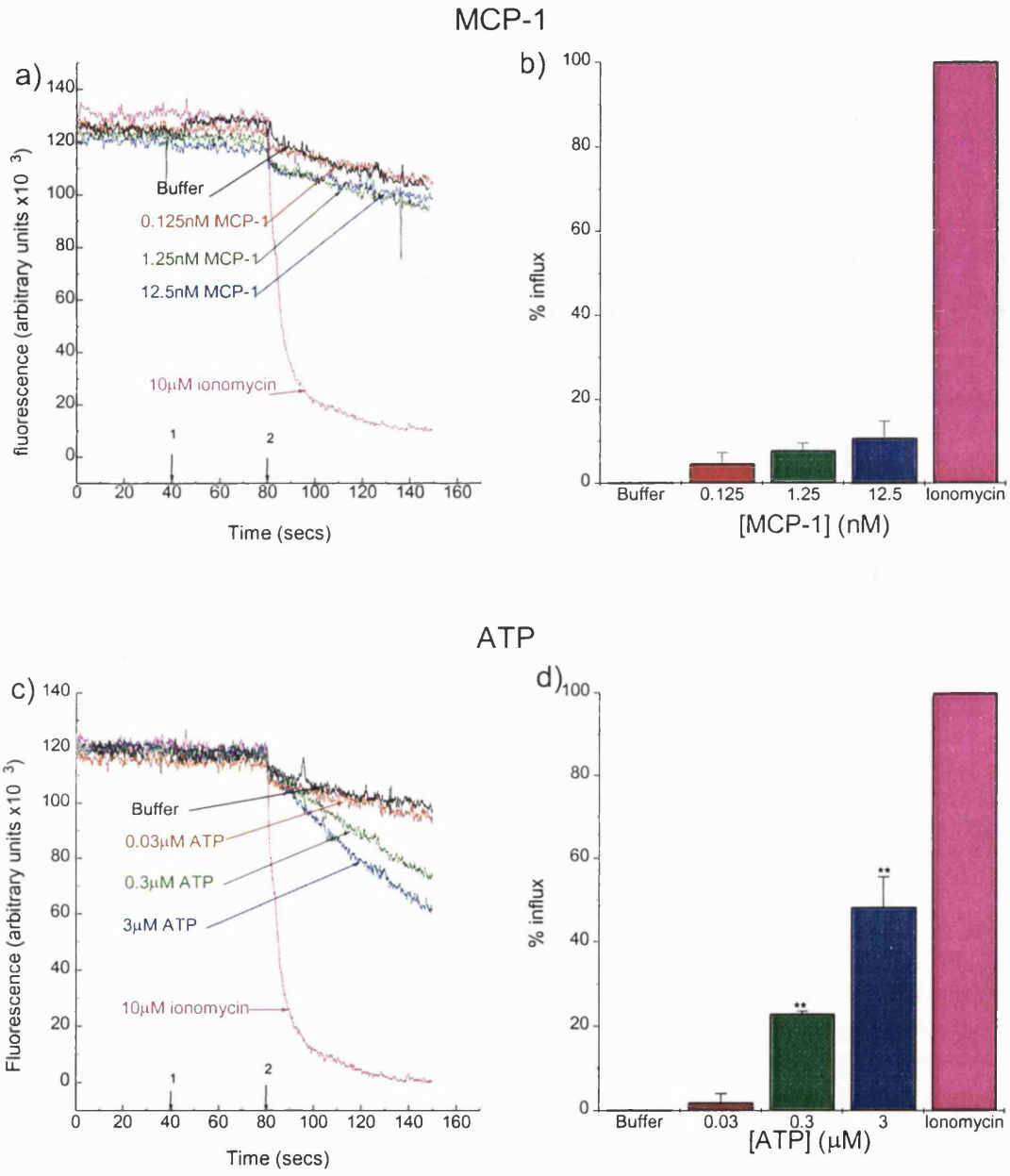


Figure 4.7 - Mn^{2+} influx in THP-1 cells in response to MCP-1 and ATP. 10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. They were also maintained in 100μM Ca^{2+} containing medium and pelleted and resuspended immediately prior to use. For all traces, buffer, ionomycin or agonist was added at 40 seconds (1) and 300μM Mn^{2+} was added 40 seconds later (2). The changes were studied at an excitation wavelength of 360nm. All traces are representative of five separate experiments. On the right hand side are the graphical representations of the Mn^{2+} influx (mean +/- s.e.m., n=5). The results are expressed as a percentage of the ionomycin influx, with the buffer control taken as zero.

4.1.3.2 Involvement of PLC in the MCP-1-induced elevation in $[Ca^{2+}]_i$

Figure 4.8 shows the effects of the PLC inhibitor U73122 (Smith *et al.* 1990) on the MCP-1-induced calcium response. U73122 completely inhibited the MCP-1-induced $[Ca^{2+}]_i$ elevation with an IC_{50} of 3.0 ± 0.34μM (n=3-5). 5 minute pre-treatment of the cells with the inactive isomer of U73122, namely U73343, had no effect on the calcium response following MCP-1 stimulation.

To prove that MCP-1 activated PLC, the effects of 12.5nM MCP-1 on IP_3 levels were investigated. The basal IP_3 levels of 8.0 ± 4.25pmol IP_3 / 10^7 cells (n=3) was significantly increased at 2 seconds post MCP-1 stimulation to 17.45 ± 4.3 pmol IP_3 / 10^7 cells (n=3) (figure 4.9b). This increase in IP_3 was at a maximum of 29.25 ± 7.3 pmol IP_3 / 10^7 cells (n=3) at 5 seconds post MCP-1 stimulation. By 60 seconds the IP_3 levels were not significantly different from the basal level. These increases in IP_3 preceded the increase in $[Ca^{2+}]_i$ detected following MCP-1 stimulation (figure 4.9a).

4.1.3.3 Role for PKC in the MCP-1-induced elevation in $[Ca^{2+}]_i$

To examine the role of PKC in the MCP-1-induced calcium response, the actions of the PKC activator PMA and the PKC inhibitor Ro31-8220-002 were investigated on the MCP-1-induced $[Ca^{2+}]_i$ rise. Figures 4.10a and b show the effects of PMA on the MCP-1-induced calcium response. 30nM PMA inhibited the calcium response by 68 ± 13.7% (n=5). In contrast, Ro31-8220-002 dose-dependently potentiated the MCP-1-induced calcium response (figure 4.10c and d). A 2 minute pre-treatment of the cells with 20μM Ro31-8220-002 induced a 44.2 ± 4.6% (n=5) increase in the response.

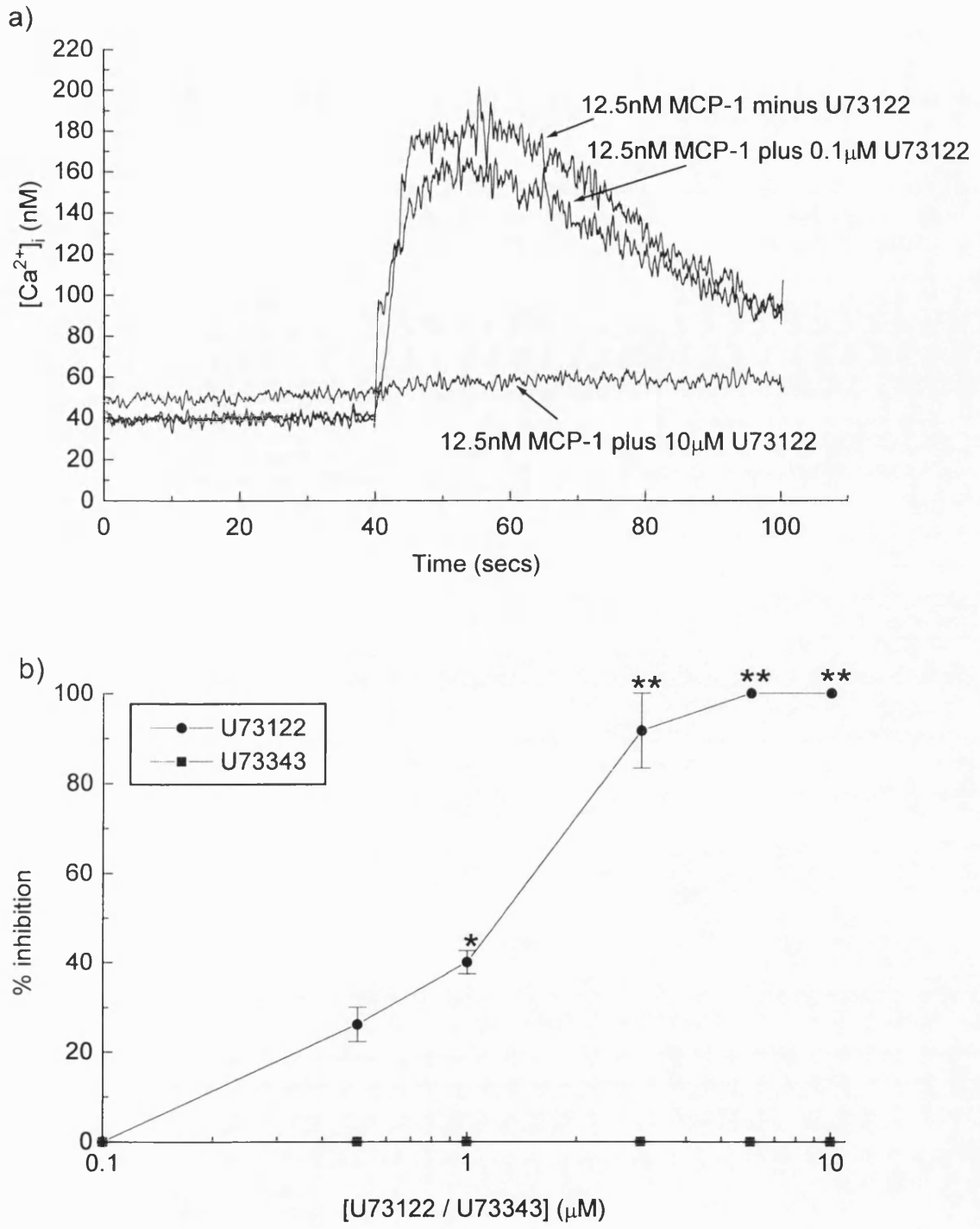


Figure 4.8 - Role of phospholipase C in the MCP-1-induced increase in $[Ca^{2+}]_i$.

10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. They were then pre-incubated with various doses of U73122/U73343 for 5 minutes prior to the addition of 12.5nM MCP-1. a) This data shows the effects of 0.1μM and 10μM U73122 on the 12.5nM MCP-1-induced increase in $[Ca^{2+}]_i$ and is representative of three to five experiments. b) The percentage inhibition of the MCP-1 response by various concentrations of U73122/U73343 (mean +/- s.e.m., n=3-5).

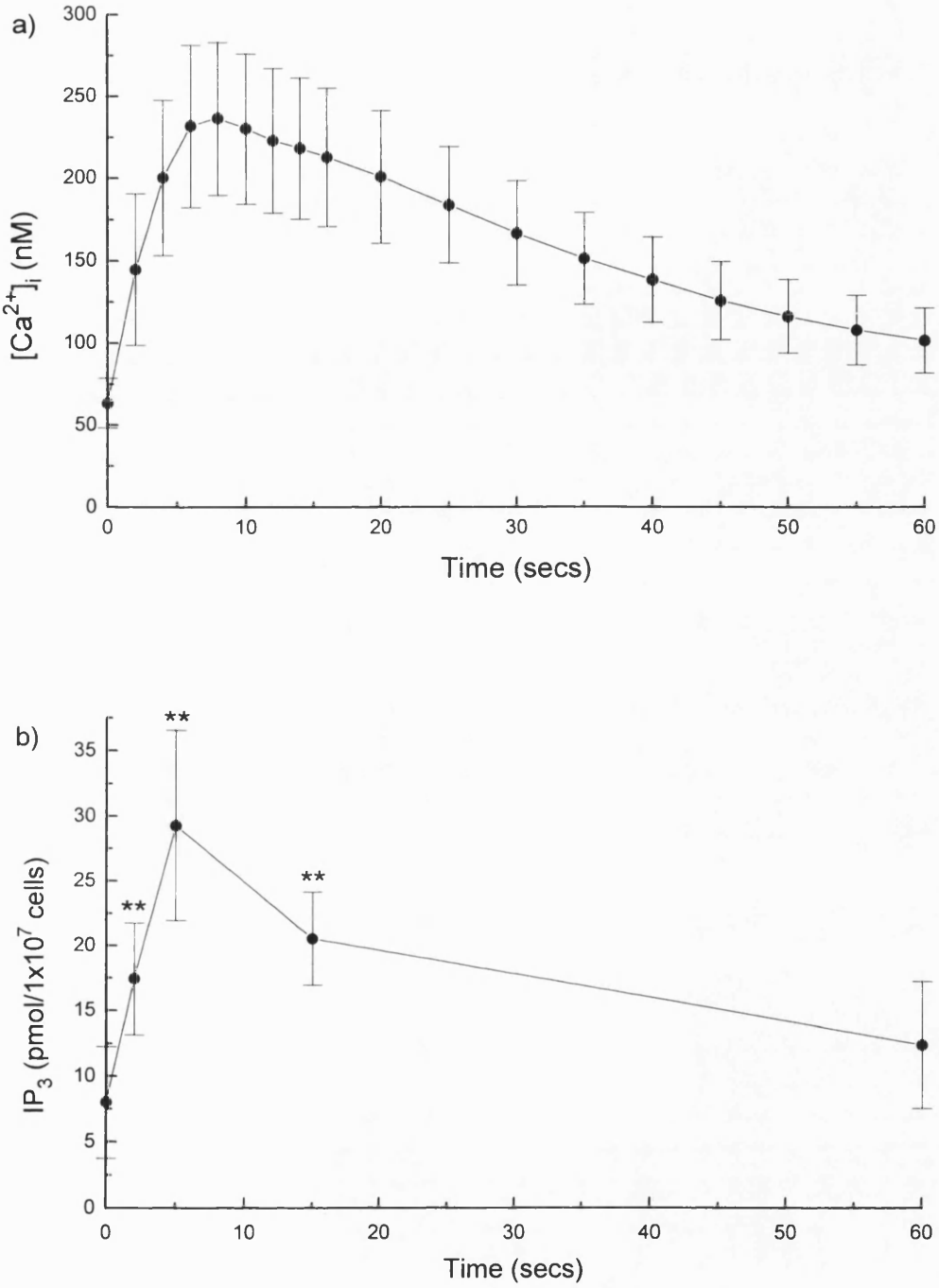


Figure 4.9 - $[Ca^{2+}]_i$ and IP_3 levels in THP-1 cells following 12.5nM MCP-1 stimulation. a) 10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. The cells were then stimulated with 12.5nM MCP-1. The mean $[Ca^{2+}]_i$ values +/- s.e.m. from six separate experiments have been plotted b)1x10^7 THP-1 cells were stimulated with 12.5nM MCP-1 and IP_3 extracted using 20% perchloric acid. The IP_3 levels were detected using a $[^3H]$-IP_3 competition assay as given in section 2.2.5. (Mean +/- s.e.m, n=3)

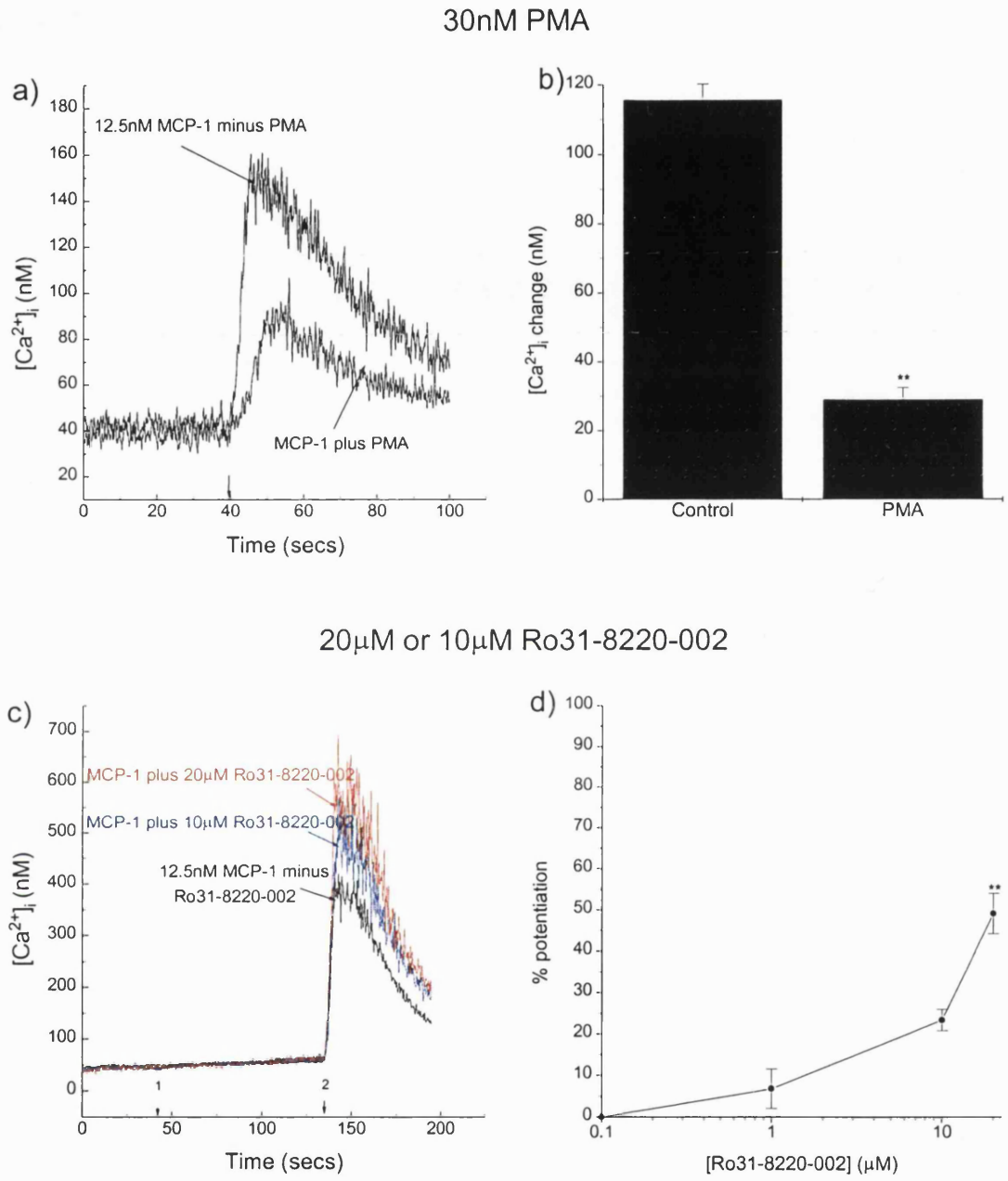


Figure 4.10 - Role of PKC in the MCP-1-induced increase in $[Ca^{2+}]_i$.

10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) 30nM PMA was incubated with the cells for 5 minutes prior to the addition of 12.5nM MCP-1. b) Graphical representation of the effects of 30nM PMA (mean+/- s.e.m., n=5). c) Various concentrations of Ro31-8220-002 were incubated with the cells for 2 minutes (1) prior to the addition of 12.5nM MCP-1 (2). d) Graphical representation to show the percentage potentiation of the MCP-1-induced calcium response in the presence of increasing concentrations of Ro31-8220-002 (mean +/- s.e.m., n=5). The calcium traces are all representative of five separate experiments.

Interestingly, the ATP-induced calcium response was also modified by the PLC inhibitor. Pre-treatment of the cells with 10μM U73122 inhibited the calcium response, although, unlike the complete inhibition observed with the MCP-1 response, only a 65.16 ± 10.8% (n=5) inhibition of the ATP response was observed (figure 4.11a and b). A role for PKC in the ATP response was also identified. Figures 4.11c and d show the effects of 30nM PMA on the ATP-induced $[Ca^{2+}]_i$ elevation. Both the initial peak and the prolonged phase of the response were inhibited by 58 ± 14.75 % (n=5) following PMA pre-treatment. Ro31-8220-002 further identified a role for PKC in the ATP-induced calcium response. The initial peak of the response was significantly increased following 20μM Ro31-8220-002 pre-incubation (figure 4.11e and f). All three compounds which appeared to affect the initial phase of the ATP response, also modified the prolonged ATP response.

4.1.3.4 Coupling of the MCP-1-induced elevation of $[Ca^{2+}]_i$ in THP-1 cells to G-proteins

Since, to date, all of the chemokine receptors have been identified as 7 transmembrane spanning, G-protein -coupled receptors, the effects of the G_i/G_o G-protein inhibitor pertussis toxin was investigated. Figure 4.12 demonstrates that pertussis toxin pre-treatment inhibited the MCP-1-induced $[Ca^{2+}]_i$ elevation in a dose-dependent manner. The IC_{50} for this inhibition was 0.58 ± 0.05ng/ml (n=3). Interestingly, pertussis toxin pre-treatment of the cells had no significant effect on the ATP-induced calcium response (figure 4.13).

Figure 4.14 demonstrates that the THP-1 cells also responded to another purinergic agonist, namely UTP. This calcium response was very comparable with the response observed upon ATP stimulation of these cells. The maximum change in $[Ca^{2+}]_i$ in response to 3μM ATP and UTP were 293 ± 43.8nM (n=5) and 396.7 ± 49.1nM (n=3) respectively.

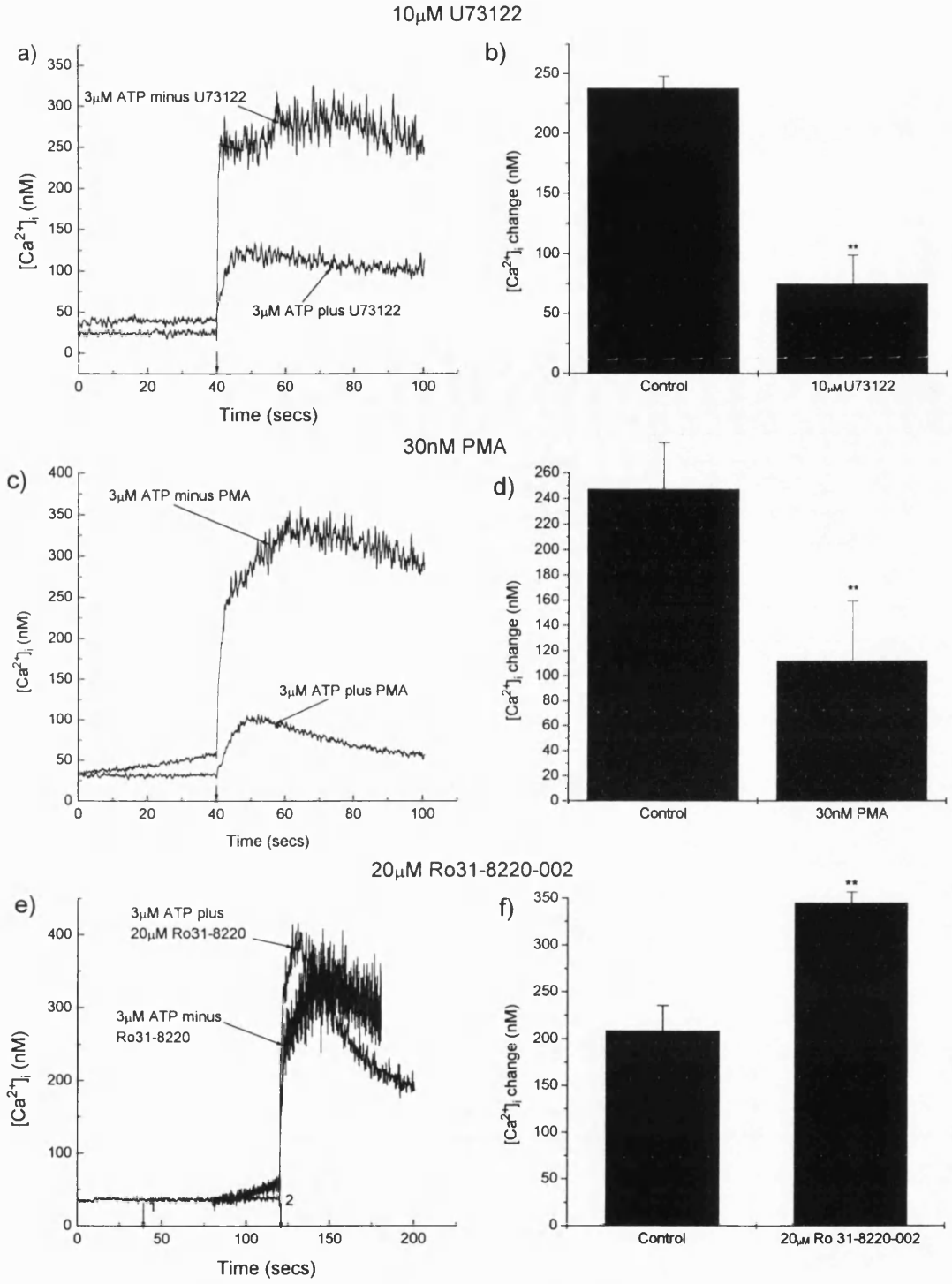


Figure 4.11 - Role of PLC and PKC in the ATP-induced rise in $[Ca^{2+}]_i$.

$10^6$$10^7$ THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The cells were incubated with 10μM U73122 for 5 minutes prior to the addition of 3μM ATP. b) Graphical representation of the effects of U73122 c) 30nM PMA was incubated with the cells for 5 minutes prior to the addition of 3μM ATP. d) Graphical representation of the effects of PMA. e) Ro31-8220-002 was incubated with the cells for 2 minutes (1) prior to the addition of 3μM ATP (2). f) Graphical representation of the effects of Ro31-8220-002 on the ATP response. The measurements were taken 5 seconds after ATP addition. The traces are all representative of five separate experiments. The graphs are all expressed as the mean $[Ca^{2+}]_i$ change +/- s.e.m., n=5.

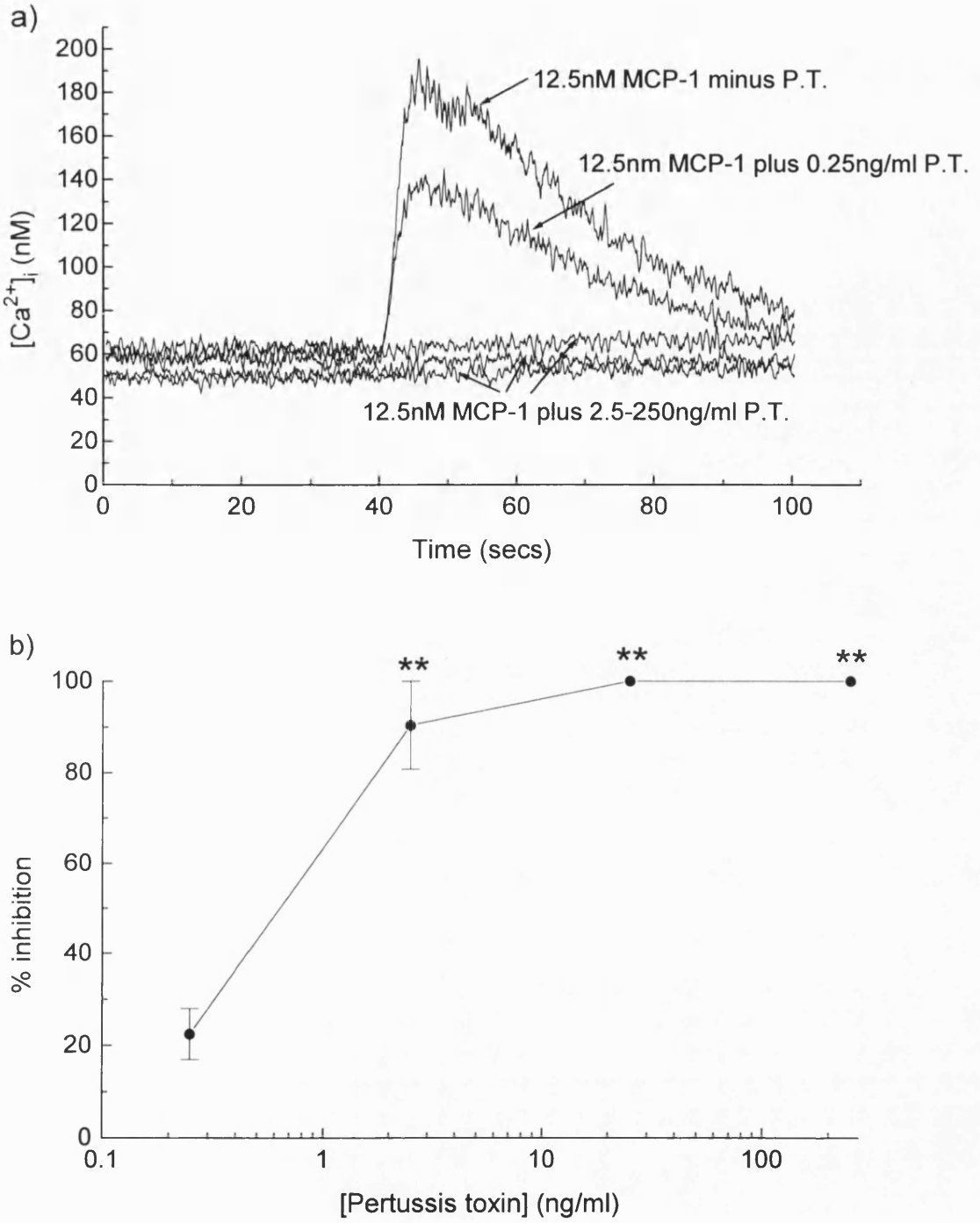


Figure 4.12 - Effects of pertussis toxin on the MCP-1-induced increase in $[Ca^{2+}]_i$. The cells were pre-treated for 16 hours with various concentrations of pertussis toxin and then 10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) This data shows the 12.5nM MCP-1-induced increases in $[Ca^{2+}]_i$ in the presence/absence of pertussis toxin. The traces are representative of three separate experiments. b) The percentage inhibition after four separate concentrations of pertussis toxin (mean +/- s.e.m, n=3).

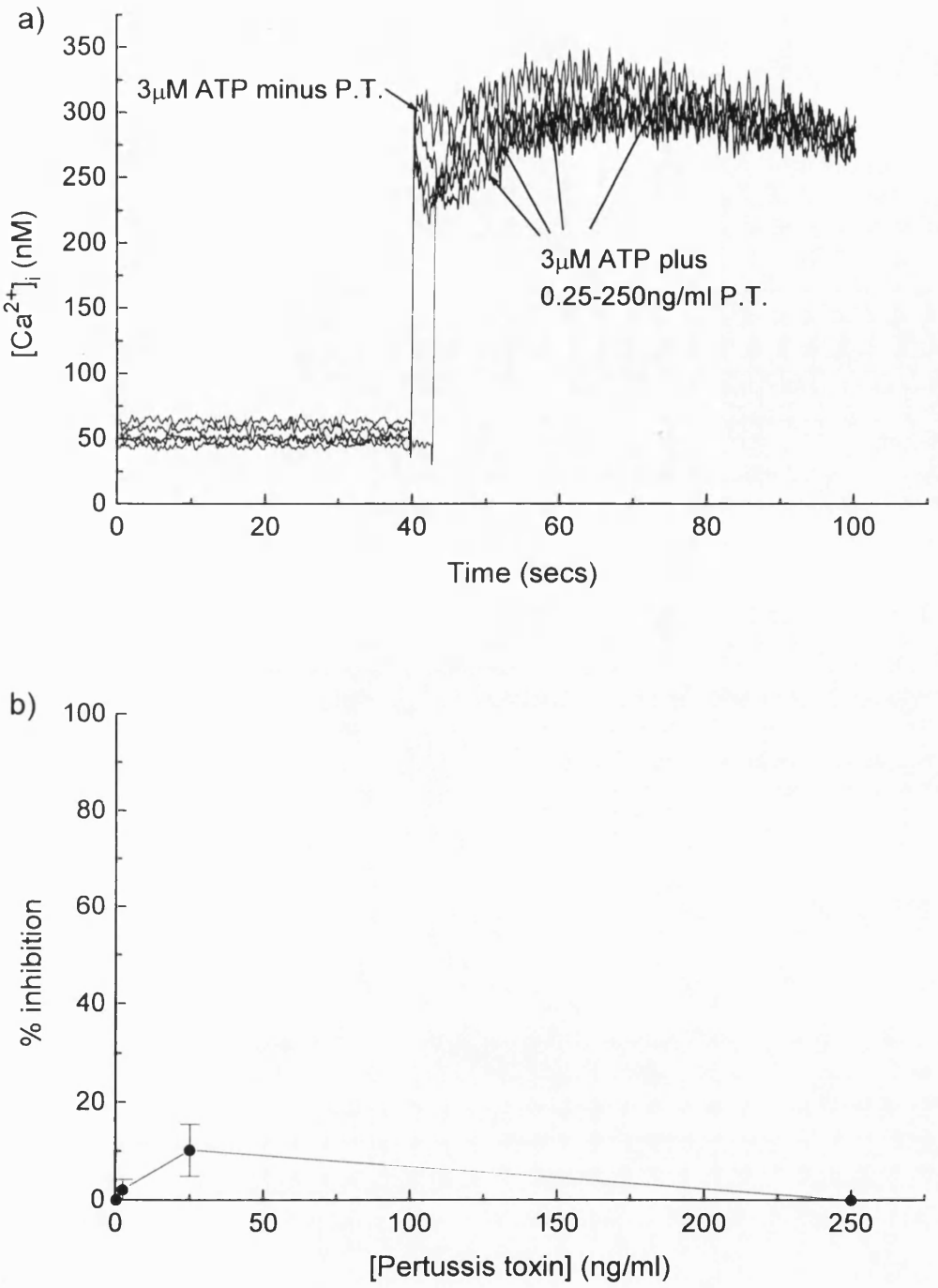


Figure 4.13 - Effects of pertussis toxin on the ATP-induced rise in $[Ca^{2+}]_i$.
THP-1 cells were pre-treated with various concentrations of pertussis toxin for 16 hours and then 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) This data shows the 3μM ATP $[Ca^{2+}]_i$ responses in the presence/absence of pertussis toxin. The traces are representative of five separate experiments. b) The percentage inhibition after three separate concentrations of pertussis toxin (n=3, mean +/- s.e.m.)

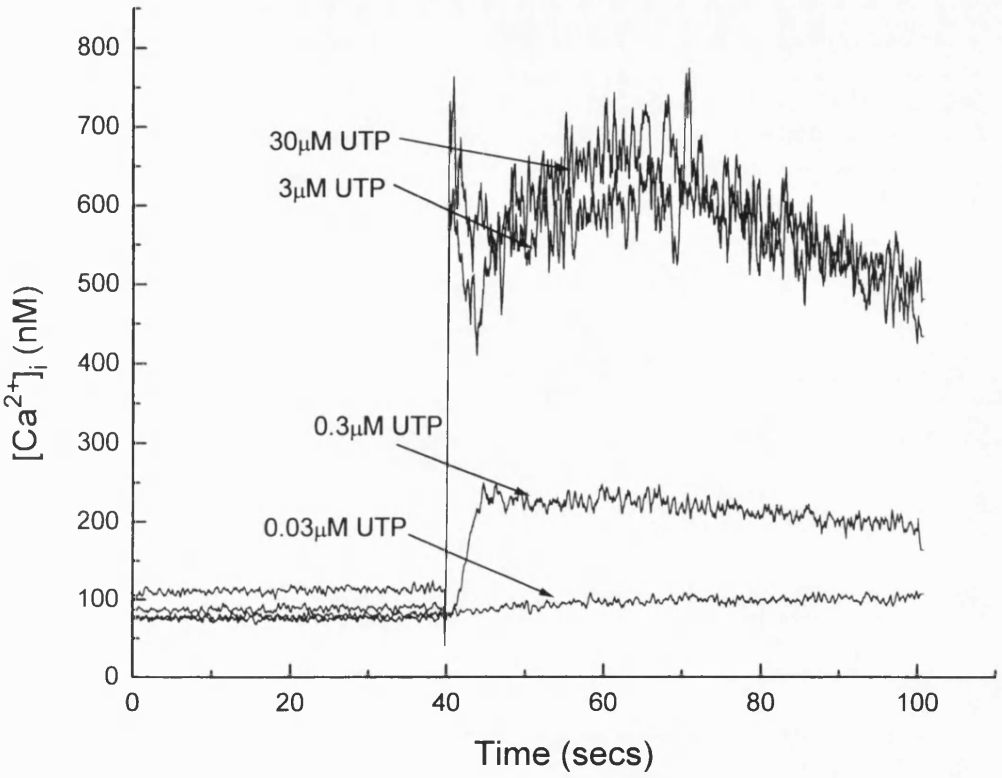


Figure 4.14 - Effects of UTP on the $[Ca^{2+}]_i$ in THP-1 cells.

10^6-10^7 THP-1 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. The traces are representative of three separate experiments.

4.2 Detection of MCP-1-induced $[Ca^{2+}]_i$ elevations in CC CKR 2A and 2B transfectants and human monocytes

4.2.1 Comparison of MCP-1-induced calcium responses

Figure 4.15a shows the effects of MCP-1 on the $[Ca^{2+}]_i$ levels in CC CKR 2A transfected cells. MCP-1 did not induce a rise in $[Ca^{2+}]_i$ at any of the concentrations tested (0.125-60nM); nor was there a calcium response induced by any other chemokines tested (n=3-5). These cells were also examined at the single cell level as well as in suspension. Again, no $[Ca^{2+}]_i$ response was detected in response to MCP-1 (n=3) (figure 4.15b). As a positive control, the effects of EGF were examined on the $[Ca^{2+}]_i$ levels. EGF has already been reported to induce increases in $[Ca^{2+}]_i$ in epithelial cells (Wahl & Carpenter, 1988). Figure 4.15c demonstrates that the CC CKR 2A transfected cells responded in a dose-dependent manner following EGF stimulation (n=3).

In contrast to the results observed with the CC CKR 2A transfected cells, the CC CKR 2B transfected cells demonstrated a dose-dependent rise in $[Ca^{2+}]_i$ in response to MCP-1 (figure 4.16). This increase in $[Ca^{2+}]_i$ was observed both with cells in suspension and at the single cell level (figure 4.16 a and b). The rise in $[Ca^{2+}]_i$ in response to other chemokines was also examined. Figure 4.16c shows that CC CKR 2B transfected cells did not respond to 60nM MIP-1α, MIP-1β, RANTES or IL-8. They did demonstrate a calcium rise in response to 60nM MCP-3, although the response was only 25.0 $\pm$ 1.6% (n=3) of that observed with 60nM MCP-1.

The untransfected HEK 293 cells responded to stimulation by 100ng/ml EGF, demonstrating an increase in $[Ca^{2+}]_i$ (figure 4.17). In contrast, MCP-1 did not produce an elevation of $[Ca^{2+}]_i$ in these cells.

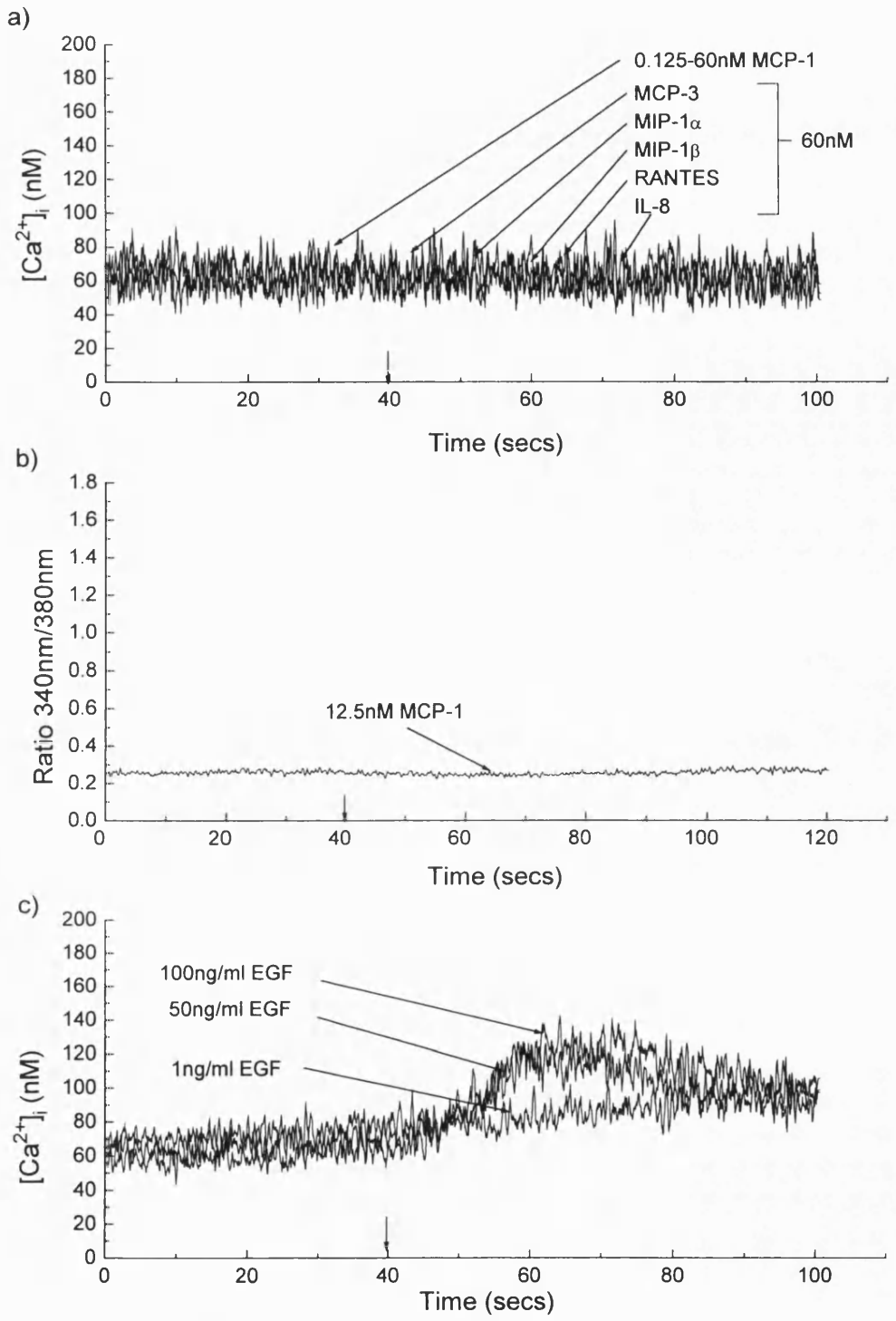


Figure 4.15 - Effects of MCP-1, related chemokines and EGF on the $[Ca^{2+}]_i$ elevation in CC CKR 2A transfected cells. 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The effect of MCP-1 and related chemokines on the CC CKR 2A transfected cells in suspension. b) The effects of MCP-1 in a small population of cells adhered onto a glass coverslip. c) The EGF responses in CC CKR 2A transfected cells in suspension. The traces are representative of at least three separate experiments.

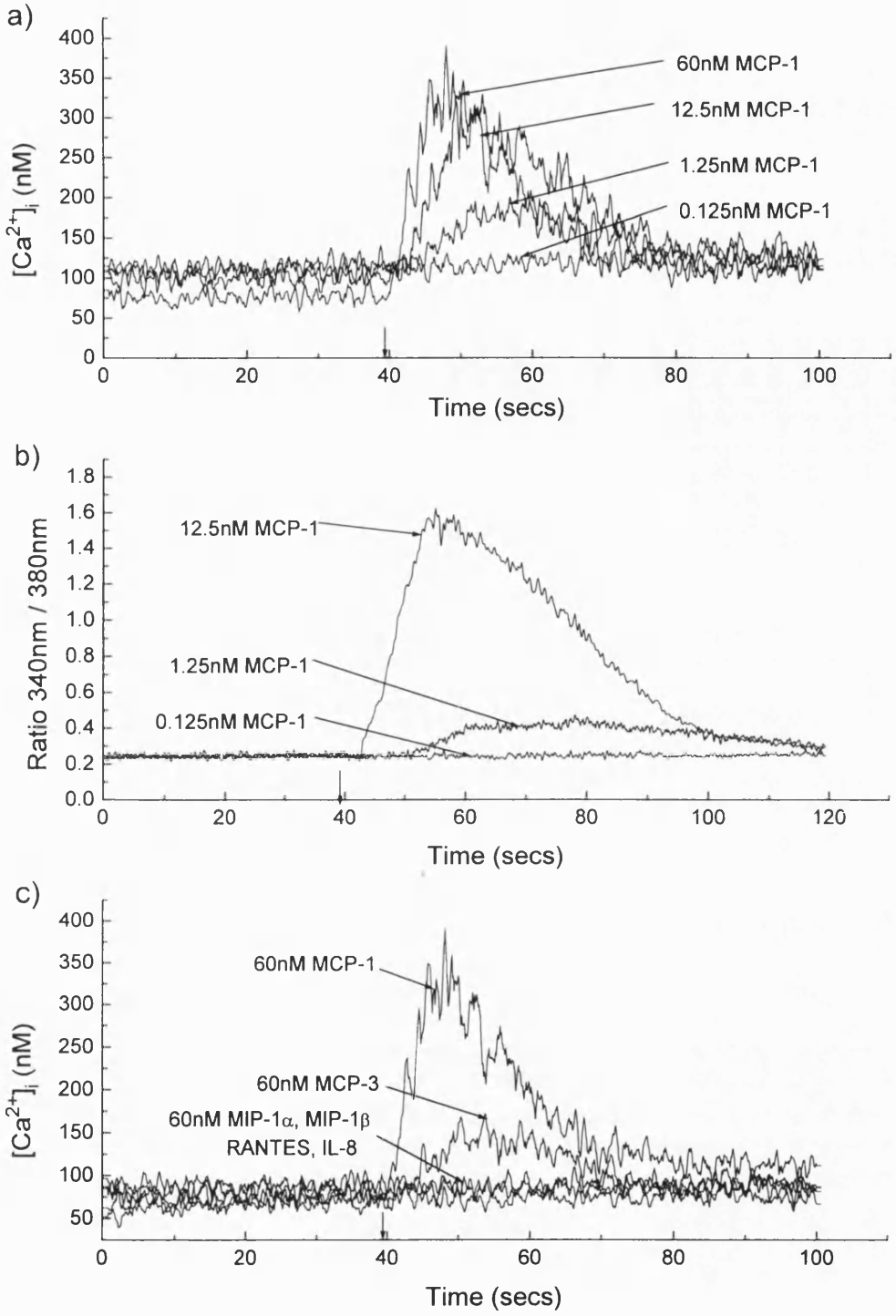


Figure 4.16 - Dose-dependent effects of MCP-1 and related chemokines on the$[Ca^{2+}]_i$ in CC CKR 2B transfected cells. 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The effect of MCP-1 on the CC CKR 2B transfected cells in suspension. b) The MCP-1 responses in a small population of cells adhered to a glass coverslip. c) The effects of 60nM MCP-1 compared to 60nM MCP-3, MIP-1α, MIP-1β, RANTES and IL-8 on cells in suspension. The traces are representative of at least three separate experiments.

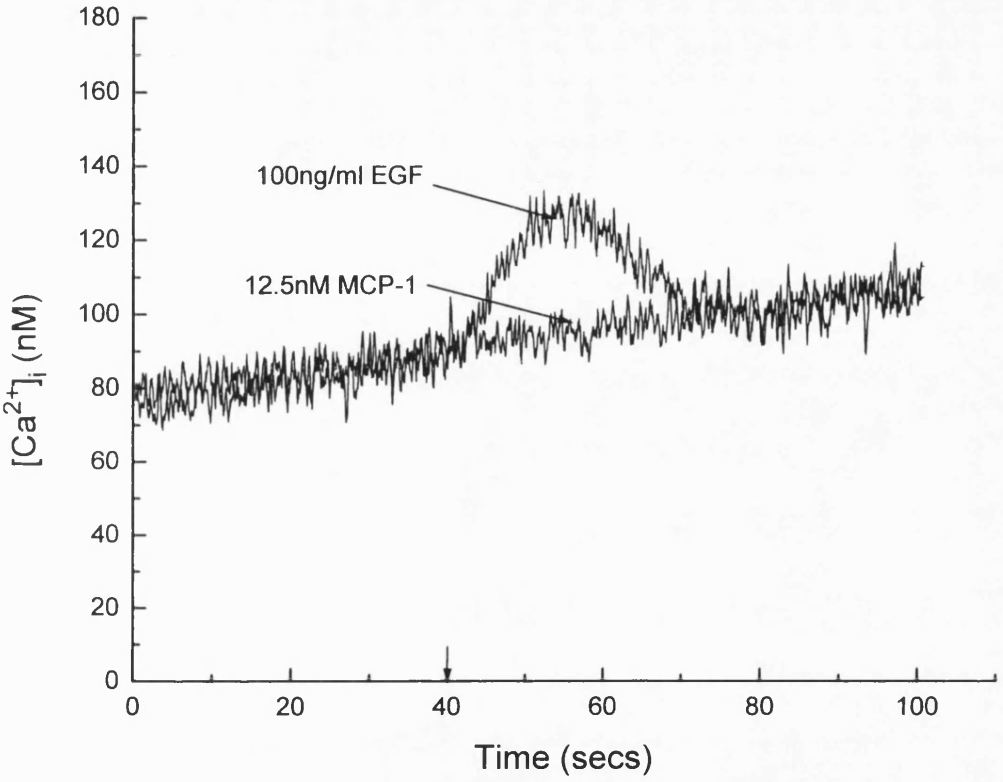


Figure 4.17 - $[Ca^{2+}]_i$ responses in untransfected HEK 293 cells in response to MCP-1 and EGF. 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4 and then stimulated with either 12.5nM MCP-1 or 100ng/ml EGF. The traces are representative of ten separate experiments.

The effect of MCP-1 on the $[Ca^{2+}]_i$ levels in human blood derived monocytes was also investigated. MCP-1 induced very similar increases in $[Ca^{2+}]_i$ in human monocytes as observed in THP-1 cells and CC CKR 2B transfected cells (figure 4.18), although, unlike the other two cell types, the MCP-1 response in human monocytes appeared to remain elevated for a longer time period (n=10). The traces observed with human monocytes demonstrated more background noise due to the number of cells used. As a result of the limited number of monocytes obtained for each experiment, only half the number of monocytes required ($1x10^6$ instead of $2x10^6$) could be used for each trace to allow a complete experiment to be performed.

4.2.2 Comparison of the source of the MCP-1-induced $[Ca^{2+}]_i$ elevation in CC CKR 2B transfected cells and human monocytes

4.2.2.1 Role for calcium influx in the increase in $[Ca^{2+}]_i$

Pre-incubation of CC CKR 2B transfectants with 1mM EGTA inhibited the $[Ca^{2+}]_i$ rise in response to MCP-1 by 53.66 ± 4.6% (n=3). The readdition of 1mM Ca^{2+} returned the response to a level observed in the absence of EGTA (figure 4.19). Figure 4.20 demonstrates the Mn^{2+} influx observed in the CC CKR 2B transfected cells following stimulation by various concentrations of MCP-1. MCP-1 induced a dose-dependent influx of Mn^{2+} in these cells. 12.5nM MCP-1 induced a Mn^{2+} influx response which was 34.03 ± 5.79% (n=3) of the response observed in the presence of digitonin (figure 4.20b).

The effects of EGTA on the MCP-1-induced $[Ca^{2+}]_i$ elevation in the human monocytes was very similar to that observed in the CC CKR 2B transfectants. 1mM EGTA inhibited this calcium response by 62.1 ± 7.2% (n=7) in human monocytes (figure 4.21). The readdition of 1mM Ca^{2+} increased the response to a similar level observed in the absence of EGTA.

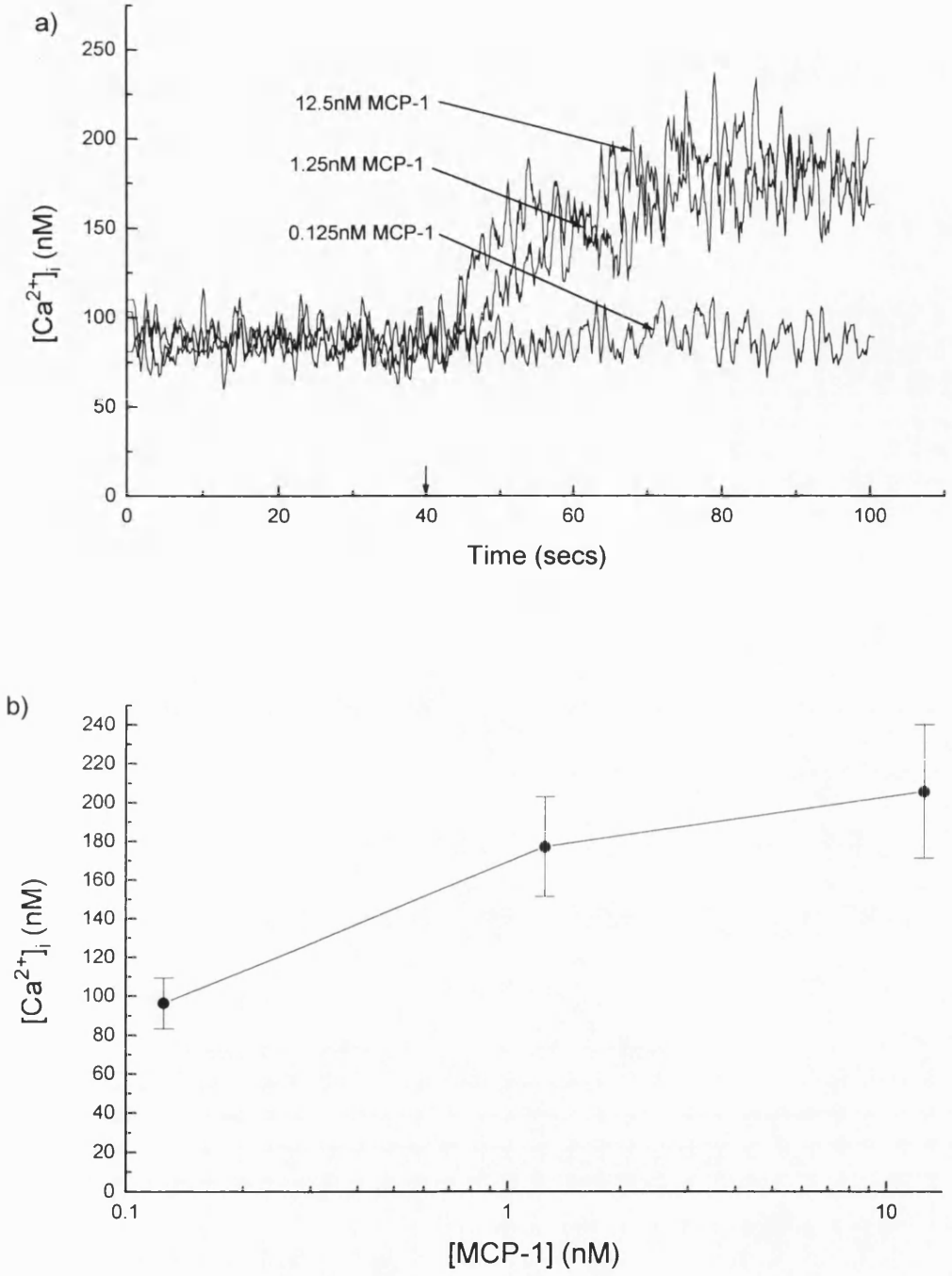


Figure 4.18 - MCP-1-induced increase in $[Ca^{2+}]_i$ in human blood-derived monocytes. The cells were purified as given in section 2.2.1.3 and 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) The increases in $[Ca^{2+}]_i$ in response to three concentrations of MCP-1. The traces are representative of ten separate experiments. b) The dose-response curve for MCP-1-induced $[Ca^{2+}]_i$ changes (mean +/- s.e.m., n=10).

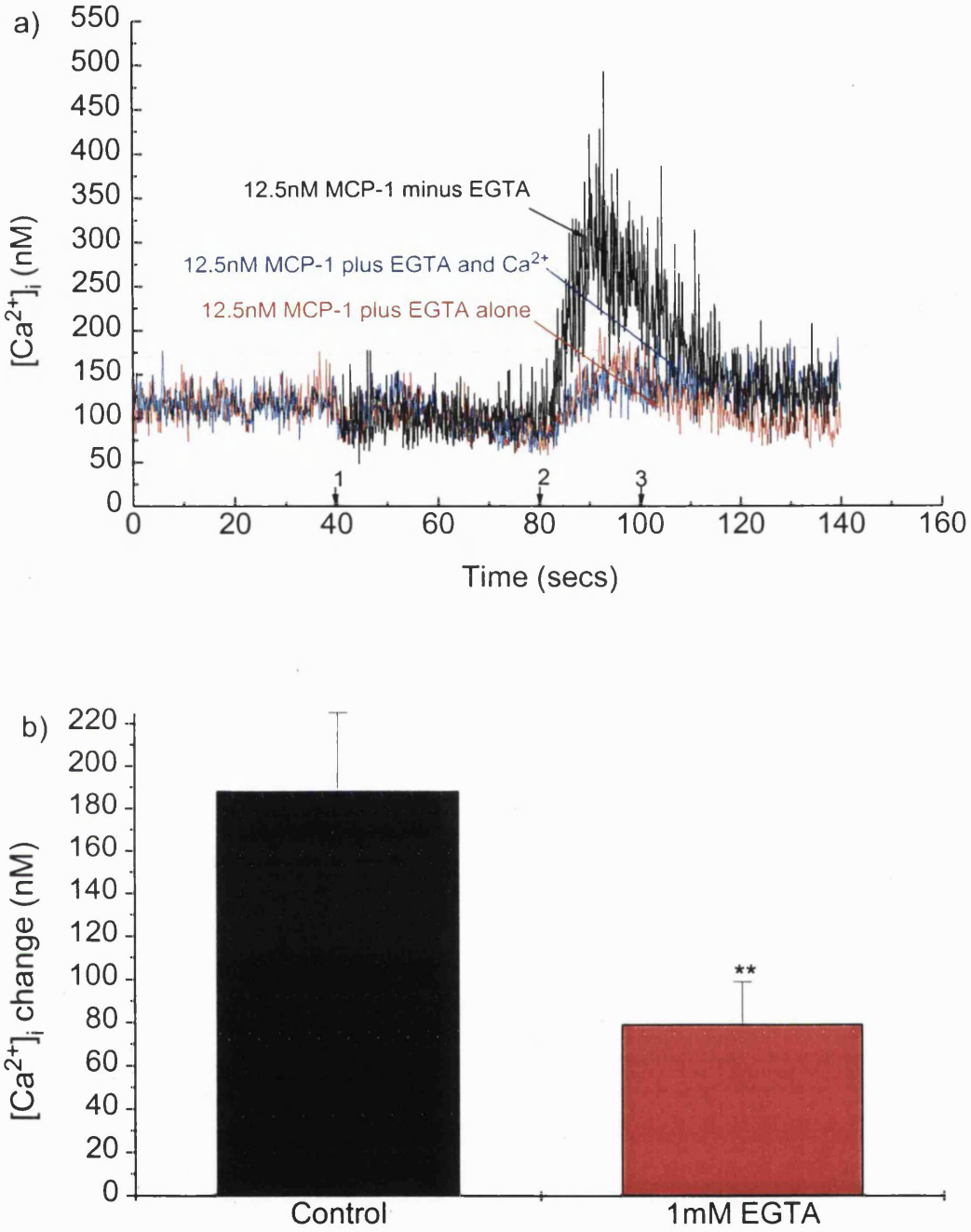


Figure 4.19 - Effects of EGTA on the MCP-1-induced increase in $[Ca^{2+}]_i$ in CC CKR 2B transfected cells. 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) 1mM EGTA was added first (1) followed by 12.5nM MCP-1 (2) 40 seconds later. The responses were subsequently followed either with or without the readdition of 1mM Ca^{2+} (3). The data is representative of three separate experiments. b) Graphical representation of the effects of EGTA. The results are expressed as the mean $[Ca^{2+}]_i$ change +/- s.e.m. of three separate expreiments.

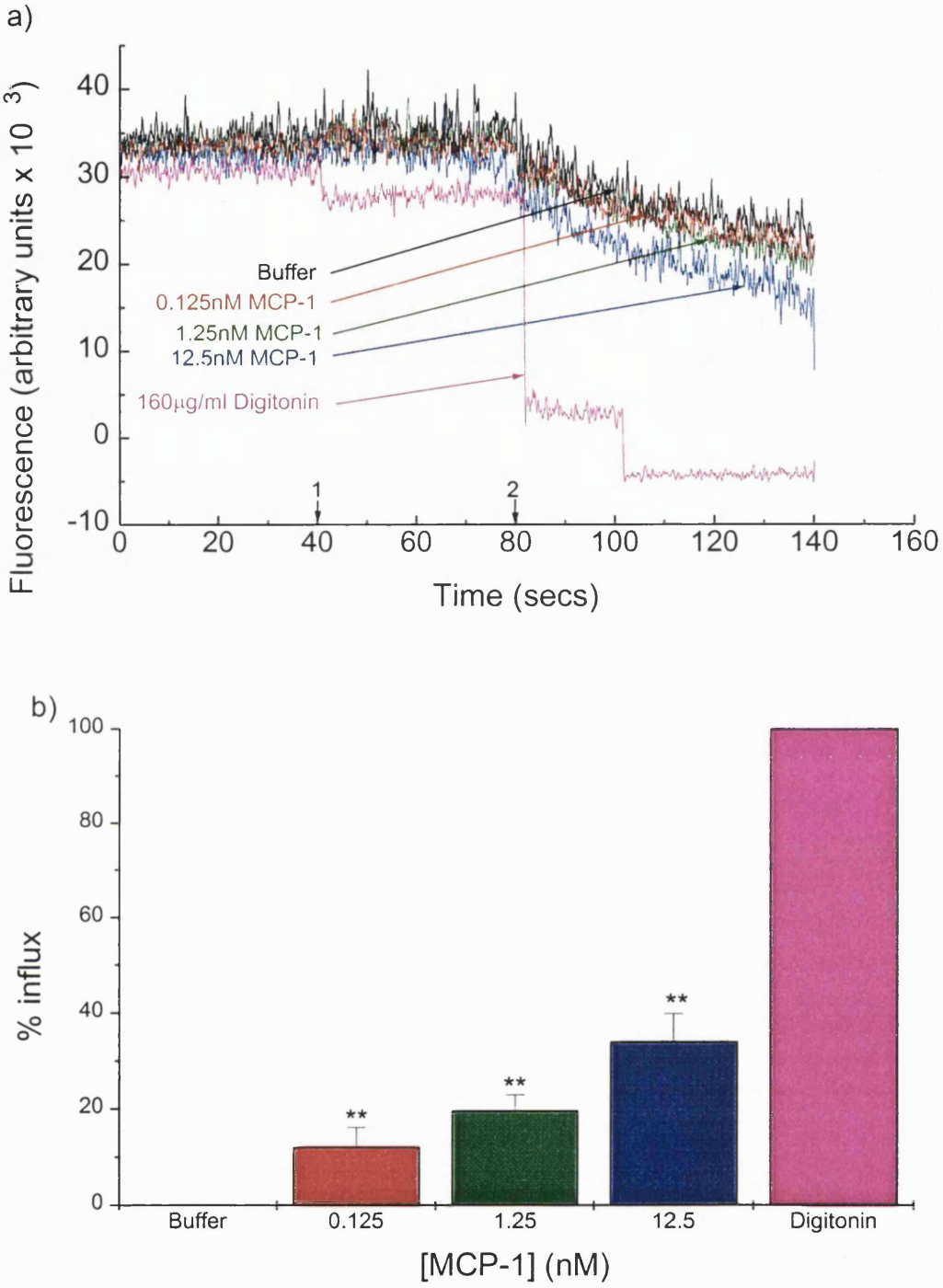


Figure 4.20 - Mn^{2+} influx in CC CKR 2B transfected cells in response to MCP-1. 10^6-10^7 cells/ml were loaded with 5µM fura-2/AM as given in section 2.2.4. They were also maintained in 100µM Ca^{2+} containing medium and pelleted and resuspended immediately prior to use. a) The buffer, ionomycin or agonist was added at 40 seconds (1) and 300µM Mn^{2+} was added 40 seconds later (2). The changes were studied at an excitation wavelength of 360nm. The data is representative of five separate experiments. b) Graphical representation of the Mn^{2+} influx (mean +/- s.e.m n=5). The results are expressed as a percentage of the ionomycin influx, with the buffer control taken as zero.

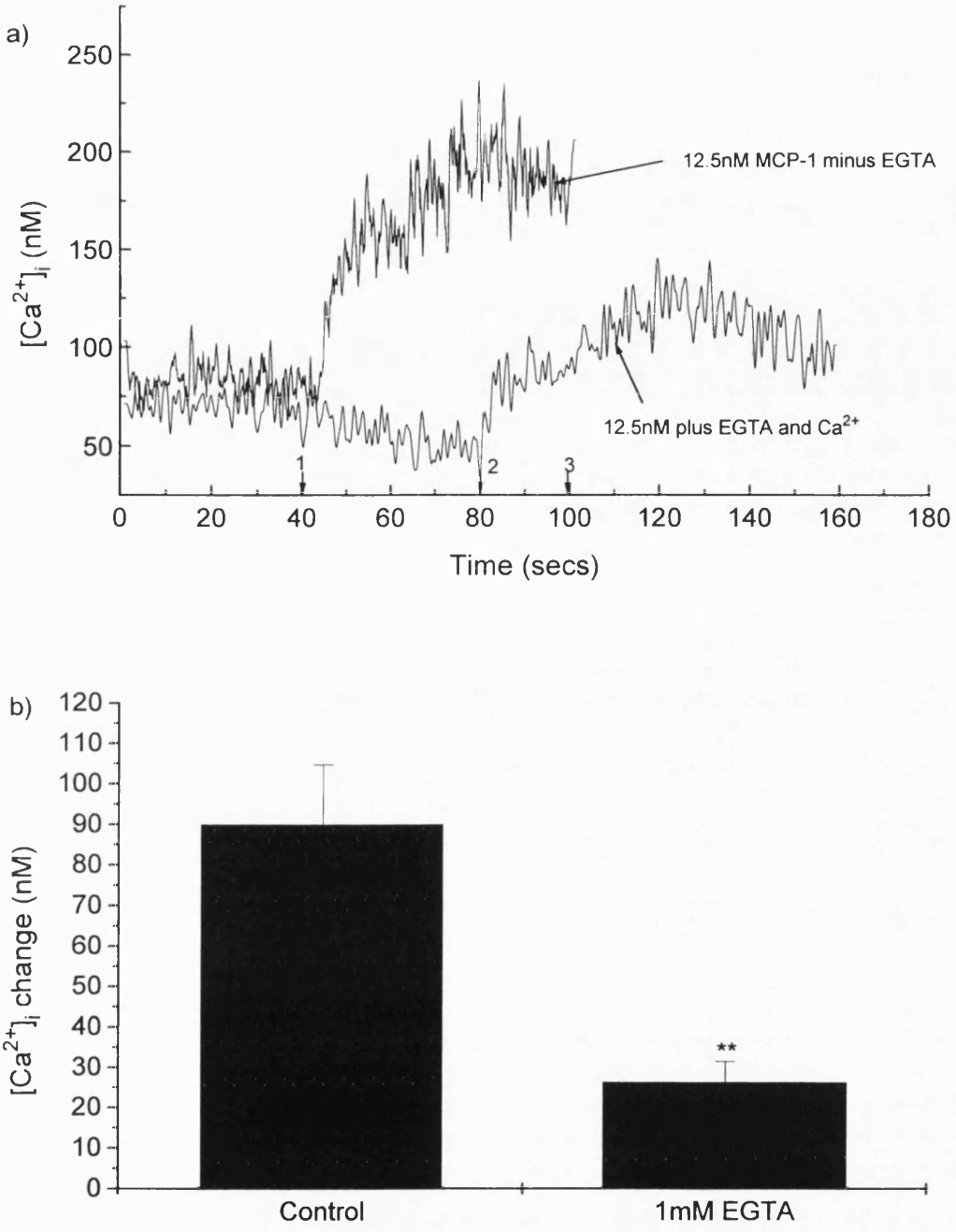


Figure 4.21 - Effects of EGTA and calcium readdition on the MCP-1-induced $[Ca^{2+}]_i$ changes in human monocytes. The cells were purified as given in section 2.2.1.3 and 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. a) 1mM EGTA was added at 40 seconds (1) followed by 12.5nM MCP-1 (2) 40 seconds later. 1mM Ca^{2+} was readded 20 seconds later (3). The traces are representative of seven separate experiments. b) Graphical representation of the effects of EGTA. The measurements were taken at the peak of the control response or 20 seconds after the addition of MCP-1 in the presence of EGTA, immediately prior to Ca^{2+} readdition. The results are expressed as the mean $[Ca^{2+}]_i$ change +/- s.e.m. from seven separate experiments.

4.2.2.2 Determination of the role of PLC in the MCP-1-induced calcium responses

The PLC inhibitor U73122 inhibited the MCP-1-induced $[Ca^{2+}]_i$ rise in the CC CKR 2B transfectants (figure 4.22a). Pre-treatment of the cells for 5 minutes with 10μM U73122 completely abrogated the calcium response. The inactive isomer of U73122, U73343, had no effect on the calcium response.

10μM U73122 also completely abrogated the calcium response induced by MCP-1 in the human monocytes (figure 4.22b). Again, U73343 demonstrated no effect.

To prove that MCP-1 activates PLC, the IP_3 levels were investigated in both the CC CKR 2A and CC CKR 2B transfected cells as well as the untransfected cells. The CC CKR 2B transfectants demonstrated a rapid and transient increase in IP_3 levels (figure 4.23b). The maximal increase from the basal level of 40.4 ± 8.3 pmol IP_3 / 10^7 cells (n=4) to 79.7 ± 15.6 pmol IP_3 /10^7 cells (n=4) was 2 seconds after 12.5nM MCP-1 stimulation. The IP_3 levels returned to basal levels by 15 seconds.

Since the CC CKR 2A transfectants did not demonstrate any calcium response to MCP-1, it was surprising to note that they demonstrated a significant increase in IP_3 levels from 51.8 ± 9.8 pmol IP_3 / 10^7 cells (n=4) to 99.3 ± 31.6 pmol IP_3 / 10^7 cells (n=4), 2 seconds after MCP-1 stimulation (figure 2.23a). This increase in IP_3 was very transient and by 5 seconds after MCP-1 stimulation, the levels had returned to basal.

The untransfected cells demonstrated no significant increase in IP_3 in response to MCP-1 at any timepoint monitored (n=4) (figure 4.23c).

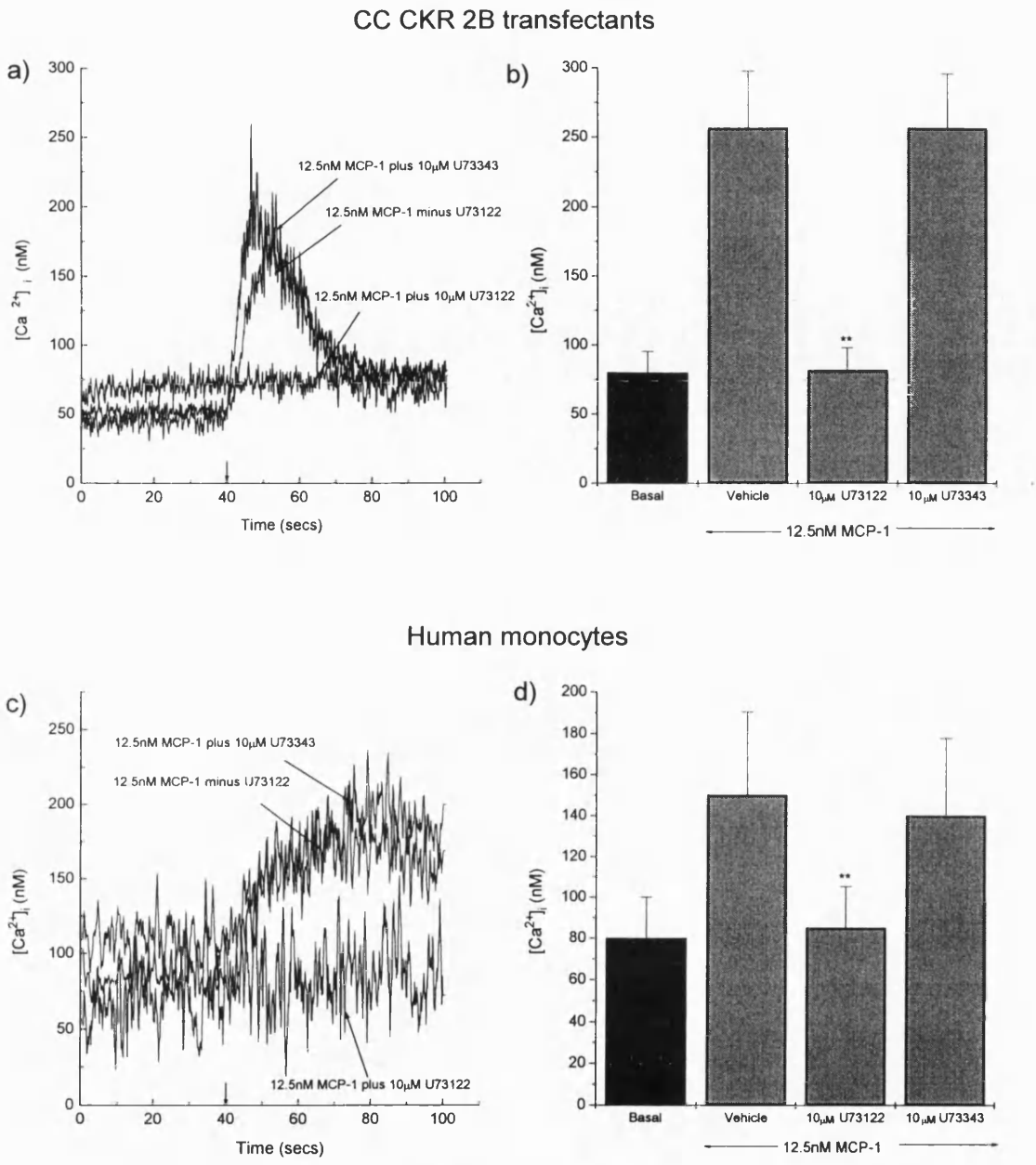


Figure 4.22 - Effects of PLC inhibition on the MCP-1-induced increases in $[Ca^{2+}]_i$ in a) and b) CC CKR 2B transfected cells and c) and d) human monocytes. The monocytes were purified as given in section 2.2.1.3. 10^6-10^7 CC CKR 2B cells or monocytes/ml were loaded with 5μM fura-2/AM as given in section 2.2.4. They were then pre-treated with either U73122 or U73343 for 5 minutes prior to 12.5nM MCP-1 addition. The traces are representative of four separate experiments. The graphical representations of the results are expressed as $[Ca^{2+}]_i$ levels in cells either unstimulated (basal), stimulated with 12.5nM MCP-1 alone or stimulated with 12.5nM MCP-1 in the presence of 10μM U73122 or U73343 (mean +/- s.e.m., n=4).

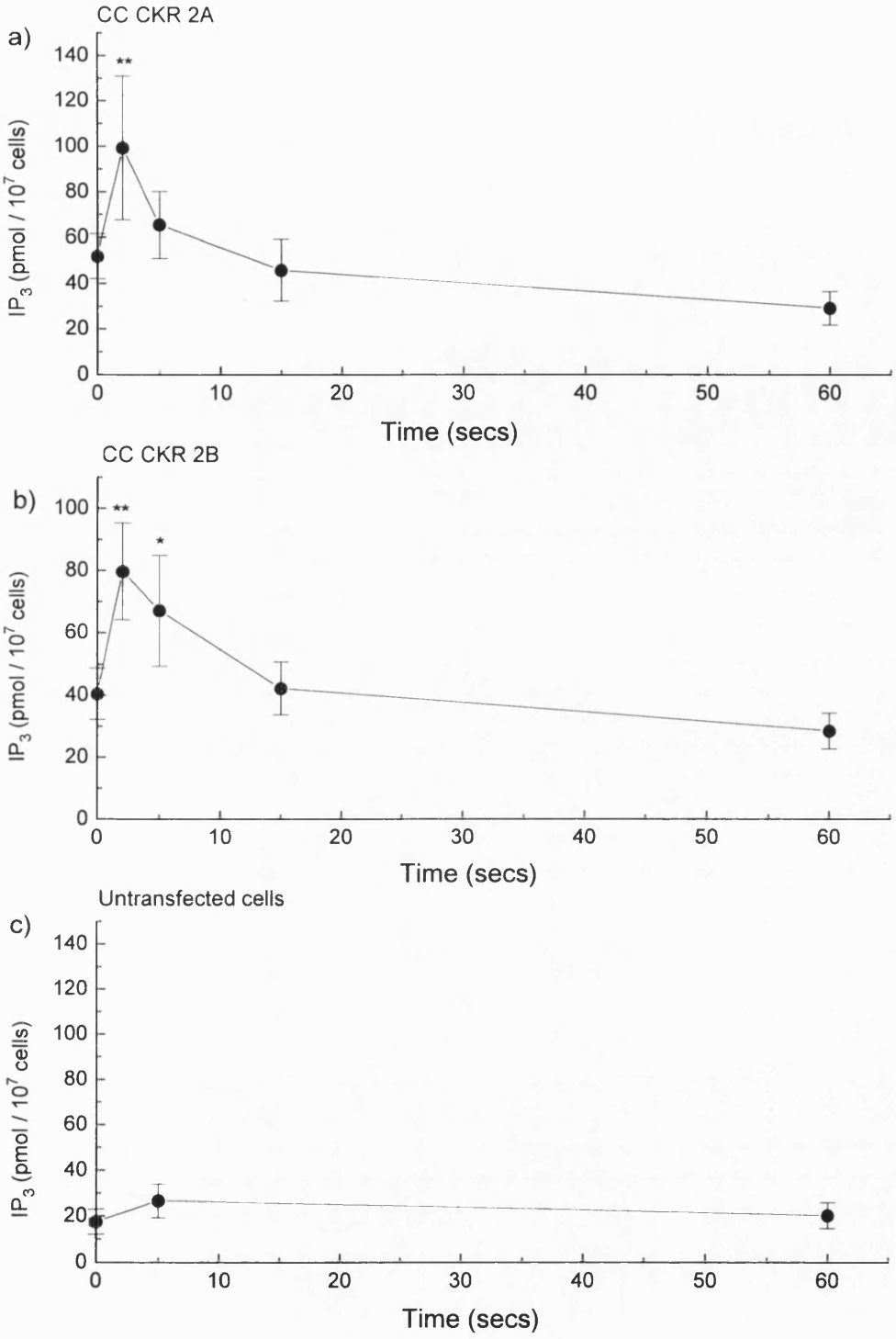


Figure 4.23 - IP_3 levels in HEK 293 cells transfected with a) the CC CKR 2A, b) the CC CKR 2B and c) untransfected cells following 12.5nM MCP-1 stimulation. $1x10^7$ cells were stimulated with 12.5nM MCP-1 and the IP_3 extracted using 20% perchloric acid. The IP_3 levels were detected using a [^{3}H]-IP_3 competition assay as given in section 2.2.5. (Mean +/- s.e.m., n=4)

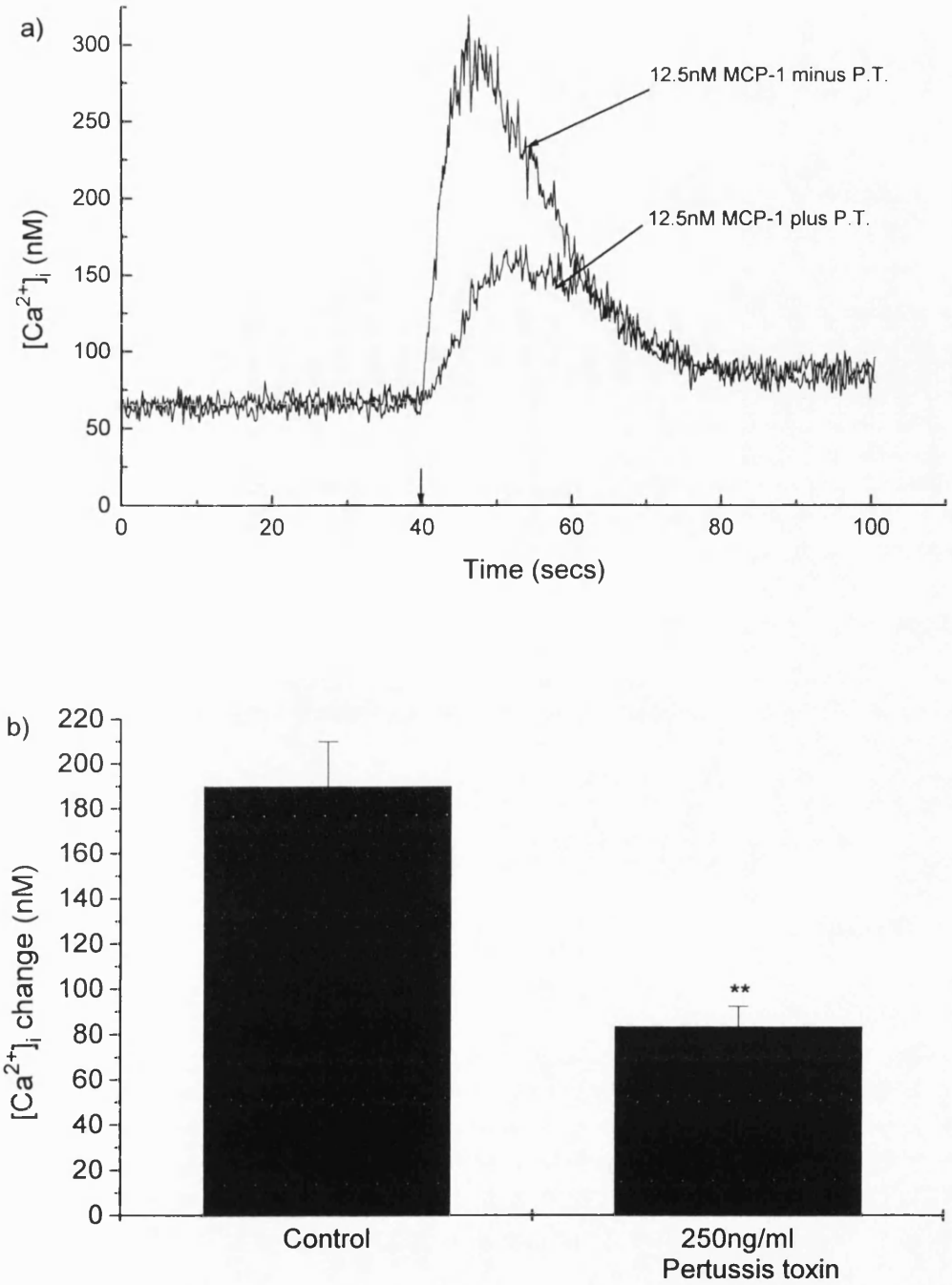


Figure 4.24 - Effects of pertussis toxin on the MCP-1-induced increase in $[Ca^{2+}]_i$ in CC CKR 2B transfected cells. a) The cells were pre-treated with 250ng/ml pertussis toxin for 16 hours and then 10^6-10^7 cells/ml were loaded with 5μM fura-2/AM as given in section 2.2.4 and stimulated with 12.5nM MCP-1. The data is representative of three separate experiments. b) Graphical representation of the effects of 250ng/ml pertussis toxin. The results are expressed as the mean $[Ca^{2+}]_i$ change +/- s.e.m. from three separate expreiments.

4.2.2.3 Coupling of the MCP-1-induced $[Ca^{2+}]_i$ response in CC CKR 2B transfectants to G-proteins

Pertussis toxin pre-treatment was used to investigate the role of G_i or G_o G-proteins in the $[Ca^{2+}]_i$ rise observed upon MCP-1 stimulation of CC CKR 2B transfected cells. The calcium response to MCP-1 in the CC CKR 2B transfectants was sensitive to pertussis toxin, as seen in figure 4.24. However, unlike the 100% inhibition observed in THP-1 cells, 250ng/ml pertussis toxin only produced a 73.9 ± 8.7% (n=3) inhibition of the calcium response.

4.3 Summary

THP-1 cells

1. During the desensitisation experiments, the loss of the $[Ca^{2+}]_i$ responses to both MCP-1 and MCP-3, no matter which of these two chemokines were administered first, demonstrated that MCP-1 and MCP-3 both bind to the same receptor. In addition, MCP-1 also produced a significant inhibition of the responses elicited by RANTES, MIP-1α and IL-8, indicating that these agonists activate a receptor also utilised by MCP-1. However, prior treatment with either RANTES, MIP-1α or IL-8 did not affect the subsequent MCP-1 response, indicating that these agonists also interact with receptors distinct from that activated by MCP-1.
2. The $[Ca^{2+}]_i$ elevation induced by MCP-1 in THP-1 cells did not require the presence of calcium in the extracellular medium, since neither EGTA nor econazole greatly inhibited the calcium response, although inhibition was observed following pre-treatment with 1mM Ni^{2+}. The lack of Mn^{2+} influx detected in these cells in response to MCP-1 further demonstrated that extracellular calcium and calcium influx are not required for the MCP-1-induced calcium response in THP-1 cells.

3. These results were in stark contrast to those observed with the ATP- induced $[Ca^{2+}]_i$ elevation. EGTA, Ni^{2+} and econazole all inhibited the prolonged phase of the ATP-induced calcium response, demonstrating the role of extracellular calcium in the maintenance of the calcium response.
4. The inhibition of the MCP-1-induced calcium response by U73122 identified the activation of PLC and its role in the elevation of $[Ca^{2+}]_i$. Proof of the activation of PLC was obtained by the time-dependent increase in the levels of IP_3 following MCP-1 stimulation, which preceded the increase in intracellular calcium.
5. A role for PLC in the initial peak of the ATP-induced calcium response was identified as U73122 inhibited this phase of the calcium response in a similar manner to that observed with the MCP-1-induced calcium response.
6. The role of PKC in the MCP-1-induced calcium response was demonstrated by both the inhibitory effects of the PKC activator PMA and the dose-dependent potentiating effects of the PKC inhibitor Ro31-8220-002.
7. PKC was also observed to play a role in the initial phase of the ATP-induced calcium response, with PMA and Ro31-8220-002 inhibiting and potentiating the initial peak of the calcium response respectively.
8. Pertussis toxin identified the activation of a G_i or G_o G-protein prior to the MCP-1-induced increase in $[Ca^{2+}]_i$, as demonstrated by the dose-dependent abrogation of the response.
9. In contrast, these G-proteins were not involved in the ATP-induced calcium response as pertussis toxin pre-treatment had no effect. Interestingly, the THP-1 cells also responded to another purinergic agonist, namely UTP, in a similar manner to that observed with ATP.

Monocytes

10. MCP-1 also induced a rise in $[Ca^{2+}]_i$ in human monocytes. The presence of extracellular calcium was required for this response as demonstrated by the

inhibition of the response by EGTA. This is in contrast to the results obtained with THP-1 cells.

11. However, the dependence of the calcium response on PLC activation was also demonstrated by the complete inhibition of the response by pre-treatment of the cells with U73122.

CC CKR 2A and 2B transfectants

12. CC CKR 2A transfectants did not demonstrate any detectable increase in $[Ca^{2+}]_i$ in response to MCP-1. In contrast, the CC CKR 2B transfectants showed a dose-dependent increase in $[Ca^{2+}]_i$.
13. The requirement of extracellular calcium for the calcium response in the CC CKR 2B transfectants was demonstrated by the inhibition of the calcium response by EGTA. Also, in CC CKR 2B transfected cells, a dose-dependent Mn^{2+} influx was detected. These were similar to the results obtained in the human monocytes
14. The calcium response in CC CKR 2B transfectants also required the activation of PLC, demonstrated by the complete abrogation of the MCP-1-induced calcium response in the presence of U73122, similar to the situation in the human monocytes.
15. The rapid increase in IP_3 in CC CKR 2B cells in response to MCP-1 proved the role for PLC in the calcium response, although, interestingly, the CC CKR 2A transfected cells also showed a small increase in IP_3 even though no calcium response was detected. The untransfected cells demonstrated no increase in either IP_3 or $[Ca^{2+}]_i$.

SECTION 5 : MCP-1-INDUCED ACTIVATION OF PI 3-KINASE

5.1 PI 3-kinase activation by MCP-1 in THP-1 cells

5.1.1 Accumulation of D-3 phosphatidylinositol lipids

Following recent publications on the activation of phosphatidylinositol 3-kinase (PI 3-kinase) in various myeloid cells such as platelets, U937 cells and neutrophils after stimulation by thrombin, ATP and fMLP respectively (Thomason *et al.* 1994)(Stephens *et al.* 1993) (Stephens *et al.* 1991), the activation of this novel pathway by MCP-1 was examined in THP-1 cells. The initial method utilised was the measurement of the accumulation of D-3 PtdIns lipids from [^{32}P]-labelled cells. Hence, after activation of the cells, lipids were extracted, deacylated and the glycerophosphoinositol derivatives of the D-3 PtdIns lipids were separated by HPLC and detected by an on-line radiodetector. Figure 5.1 shows representative HPLC traces of unstimulated and stimulated cells. In figure 5.1a, the positions of the main phospholipid products in unstimulated cells are marked by the arrows. In figure 5.1b, the cells have been stimulated with 180nM MCP-1 for 30 seconds and the position of the three PI 3-kinase products, namely PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are indicated by arrows. The positions of the various phospholipid products were compared to standards analysed at the same time, the results obtained by Dr. Stephen Ward using the same equipment and published data (Stephens *et al.* 1991) (Traynor-Kaplan *et al.* 1988) (Cheatham *et al.* 1994). The stimulation of THP-1 cells by MCP-1 induced an increase in both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 but no notable increases in PtdIns(3)P. Figure 5.2b and c shows the time-dependent increases in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. 180nM MCP-1 was used to study the activation kinetics of PI 3-kinase by MCP-1. The increase in PtdIns(3,4,5)P_3 was fairly rapid but transient when compared to the increase in PtdIns(3,4)P_2, which was over a more sustained period with slower kinetics.

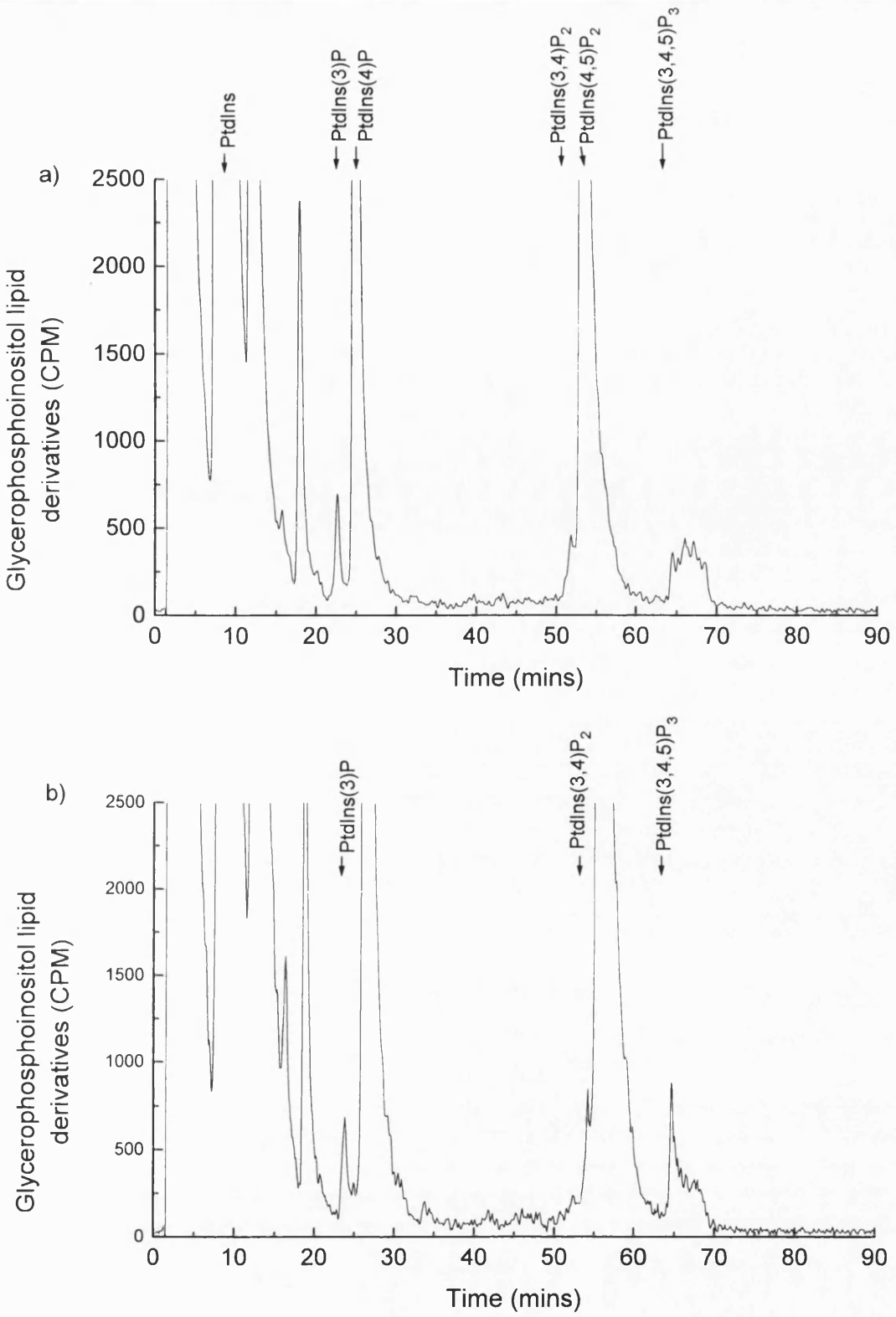


Figure 5.1 - Typical HLPC traces obtained from a) unstimulated cells and b) cells stimulated with 180nM MCP-1 for 30 seconds following phospholipid extraction and deacylation. The sample preparation was as given in section 2.2.6. The positions of the main phospholipid products are as marked.

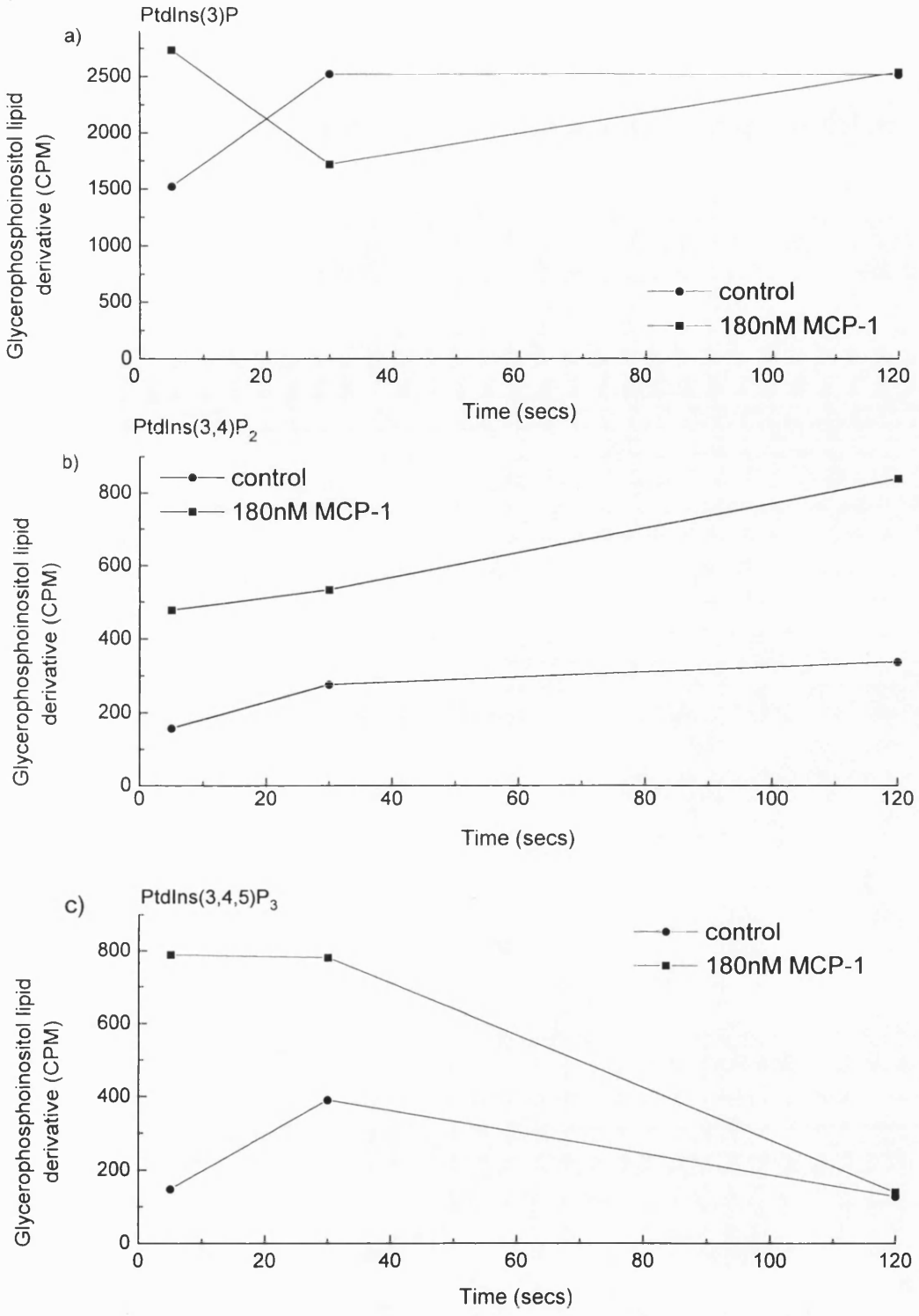


Figure 5.2 - Timecourse of a) PtdIns(3)P, b) PtdIns(3,4)P_2 and c) PtdIns(3,4,5)P_3 changes in THP-1 cells in response to 180nM MCP-1 stimulation. 1x10^7 [^{32}P] labelled cells were stimulated for various times with either vehicle or 180nM MCP-1 and then extracted, deacylated and analysed as given in section 2.2.6. The data is representative of at least three separate experiments.

Since the accumulation of PtdIns(3,4,5)P_3 was optimal at 30 seconds, this timepoint was chosen for the studies on PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 accumulation following three concentrations of MCP-1. The MCP-1-induced accumulation of PtdIns(3,4,5)P_3 exhibited bell-shaped characteristics with the maximum being at 60nM MCP-1 (figure 5,3a). In contrast, the increase in PtdIns(3,4)P_2 was maximum at 180nM MCP-1 (figure 5.3b). Only a very small increase in PtdIns(3)P was observed following 12.5nM MCP-1 stimulation of these cells (figure 5.3a).

5.1.1.1 Identification of the PI 3-kinase sub-type activated by MCP-1

Since several isoforms of PI 3-kinase have been identified, it was necessary to determine which isoform is activated following MCP-1 stimulation. Hence, the pharmacological agents pertussis toxin and wortmannin were employed. Figure 5.4a shows the effects of 16 hours pre-treatment with pertussis toxin on the accumulation of PtdIns(3,4,5)P_3. Pertussis toxin almost completely abrogated the MCP-1-induced increase in PtdIns(3,4,5)P_3. In contrast, pre-incubation of the cells for 5 minutes with 100nM wortmannin had no effect on the MCP-1-induced accumulation of PtdIns(3,4,5)P_3 (figure 5.4b).

5.1.2 Determination of *in vitro* lipid kinase activity after MCP-1 stimulation

The second method of determining PI 3-kinase activation following MCP-1 stimulation was using an antibody to the regulatory p85 subunit of the PTK/SH2-coupled PI 3-kinase which was coupled to protein G sepharose beads. These immunoprecipitates were then incubated with a PtdIns substrate in the presence of [γ-^{32}P]-ATP. This *in vitro* activity resulted in the formation of radiolabelled PtdIns(3)P which was detected using TLC and autoradiography.

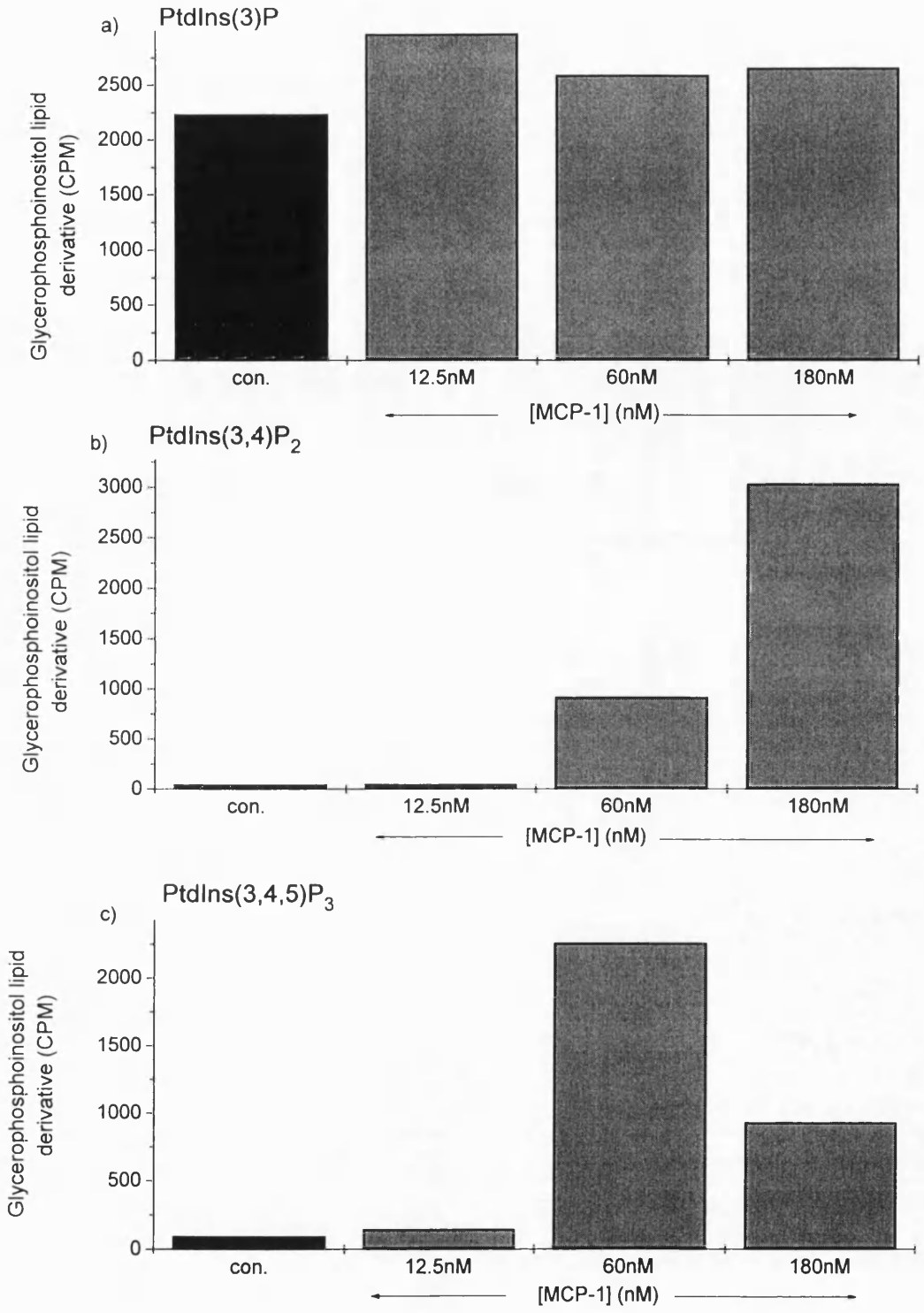


Figure 5.3 - Concentration dependent changes in a) PtdIns(3)P, b) PtdIns(3,4)P_2 and c) PtdIns(3,4,5)P_3 in THP-1 cells following a 30 second incubation with various concentrations of MCP-1. $1x10^7$ [^{32}P] labelled cells were stimulated for 30 seconds and then the samples extracted, deacylated and analysed using HPLC as given in section 2.2.6. The data is representative of at least three separate experiments.

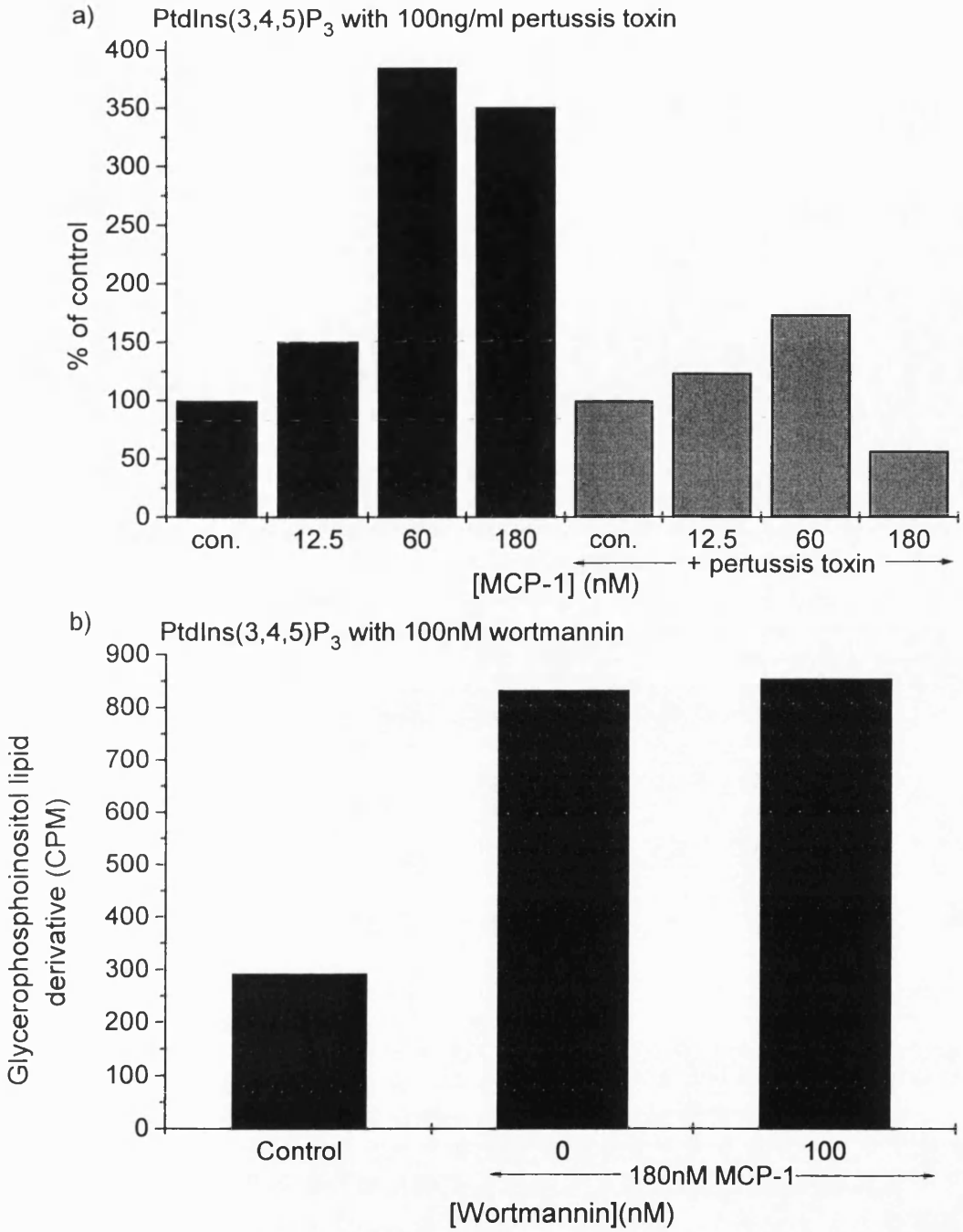


Figure 5.4 - Effects of pertussis toxin and wortmannin on the MCP-1-induced changes in PtdIns(3,4,5)P_3 in THP-1 cells. a) The cells were pre-treated with vehicle (■) or 100ng/ml pertussis toxin(▩) for 16 hours prior to the labelling of the cells as given in section 2.2.6. $1x10^7$ [^{32}P] labelled cells were then stimulated with three concentrations of MCP-1 for 30 seconds and extracted, deacylated and analysed by HPLC as given. The data is representative of at least four separate experiments and is presented as the percentage of unstimulated control. b) $1x10^7$[^{32}P] labelled cells were pre-treated with 100nM wortmannin for 5 minutes prior to stimulation by 180nM MCP-1 for 30 seconds. The samples were then extracted and deacylated as given. The data is representative of at least three separate experiments and is shown as actual CPM values.

Figure 5.5 shows the time-course of effects of 180nM MCP-1 on the generation of PtdIns(3)P from the immunoprecipitated PI 3-kinase activity. There was a time-dependent increase in this product which became notable at 60 seconds and increased up to 300 seconds. This increase in immunoprecipitated PI 3-kinase activity was also dose-dependent, as demonstrated in figure 5.6, in which the cells were stimulated with various concentrations of MCP-1 for 120 seconds. This dose-dependent increase was maximal at 300nM MCP-1, although there was a notable increase at concentrations as low as 1.25nM. Some basal activity of this enzyme was also observed.

5.1.2.1 Further characterisation of the immunoprecipitated PI 3-kinase activity stimulated by MCP-1

To further determine the nature of the lipid kinase activity in the p85 immunoprecipitates after MCP-1 stimulation, the PI 3-kinase inhibitor wortmannin, or pertussis toxin were employed. Figure 5.7 demonstrates that wortmannin produced a dose-dependent inhibition of the immunoprecipitated PI 3-kinase activity stimulated by MCP-1. The IC_{50} for this inhibition was between 0.1-1nM.

In contrast to the results obtained with wortmannin, pre-treatment of the cells for 16 hours with various concentrations of pertussis toxin (0.1-100ng/ml) had no effect on the immunoprecipitated PI 3-kinase activity stimulated by MCP-1 (figure 5.8).

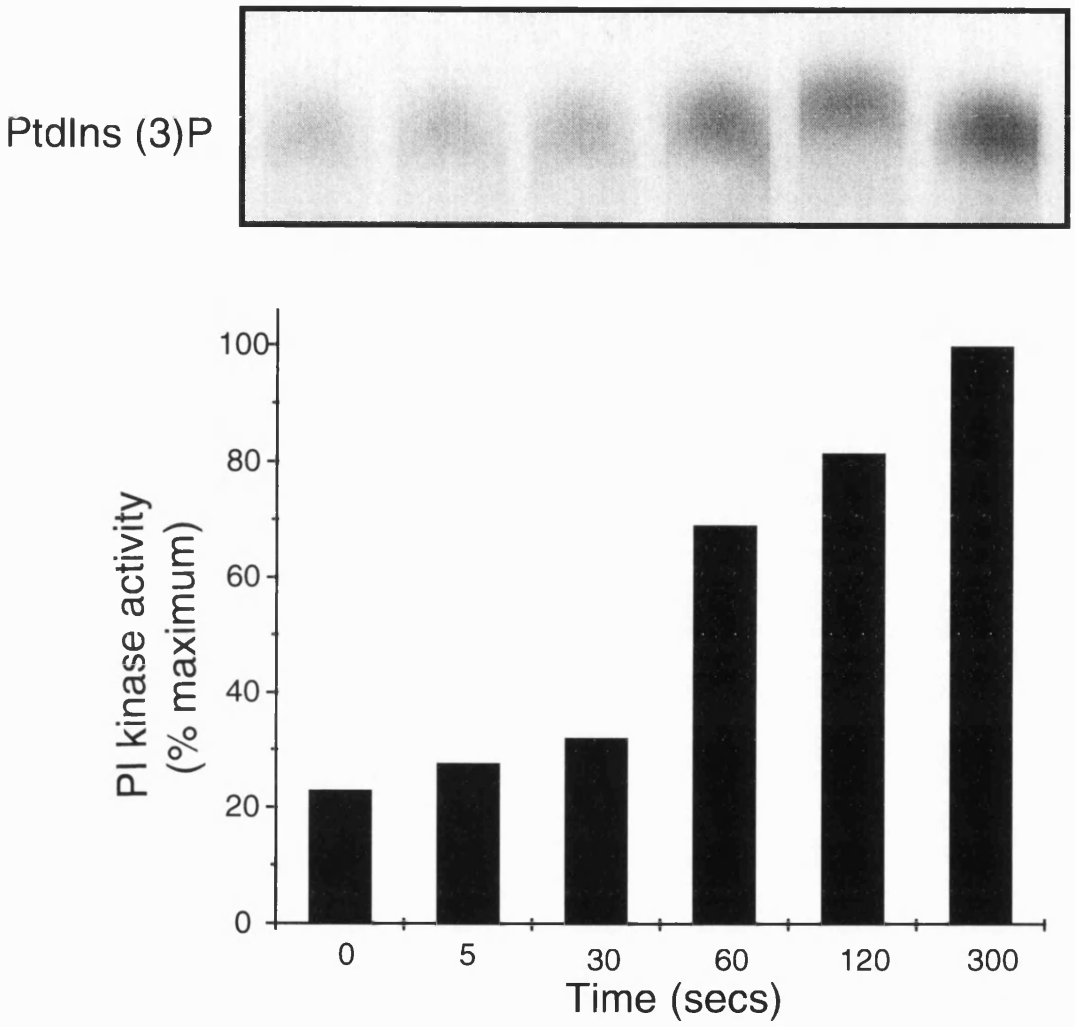


Figure 5.5 - *In vitro* lipid kinase assay of THP-1 cells stimulated for various times with MCP-1. $1x10^7$ cells were stimulated for various times with 180nM MCP-1 and then the PI 3-kinase activity was immunoprecipitated from the cell lysates using anti-p85 antibody coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned image of the autoradiograph as well as the densitometric analysis, expressing the results as PI kinase activity as a percentage of the maximum. The results are representative of at least three separate experiments.

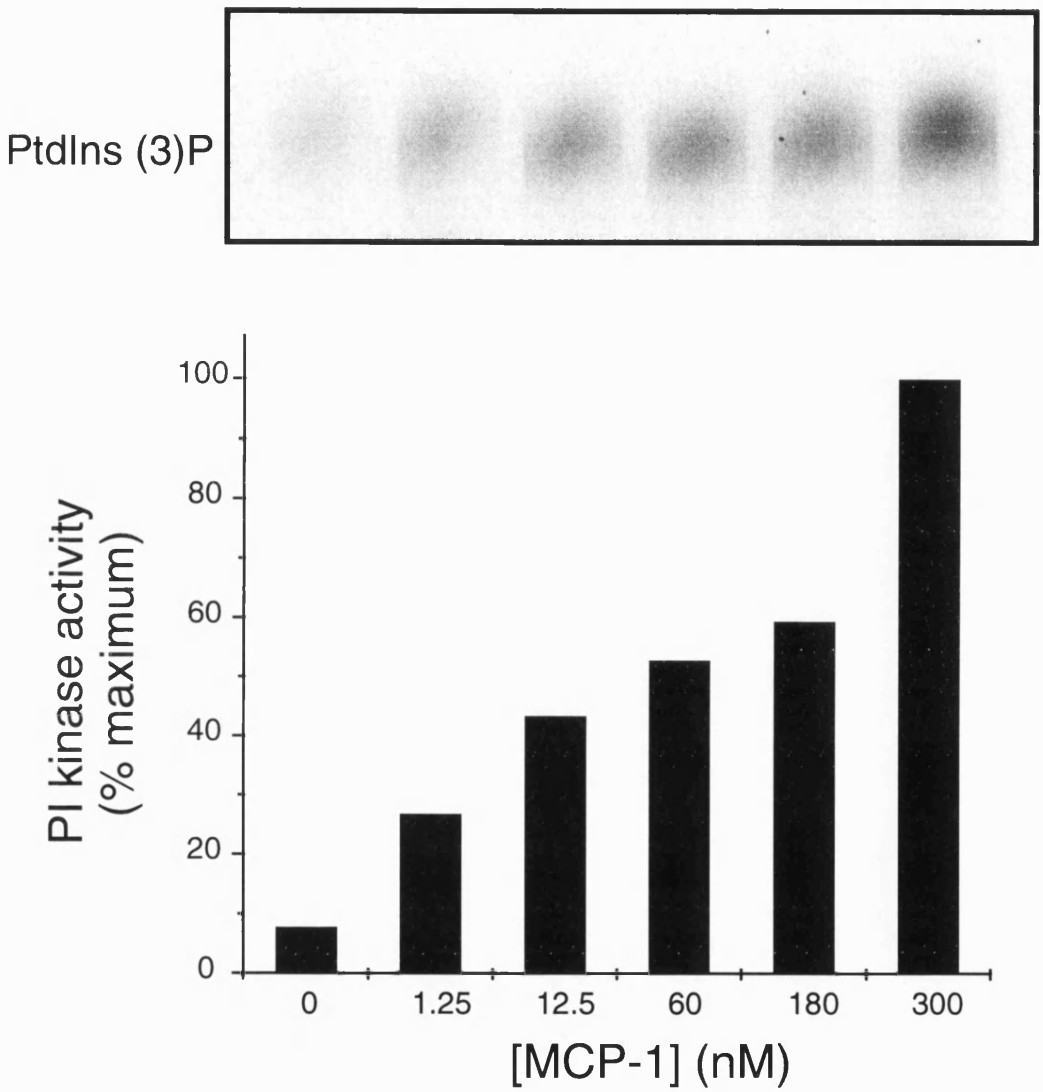


Figure 5.6 - *In vitro* lipid kinase assay of THP-1 cells stimulated with various concentrations of MCP-1. $1x10^7$ cells were stimulated with various concentrations of MCP-1 for 120 seconds and then the PI 3-kinase activity was immunoprecipitated from the cell lysates using anti-p85 antibody coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned Image of the autoradiograph as well as the densitometric analysis expressing the results as PI kinase activity as a percentage of the maximum. The data is representative of at least three separate experiments.

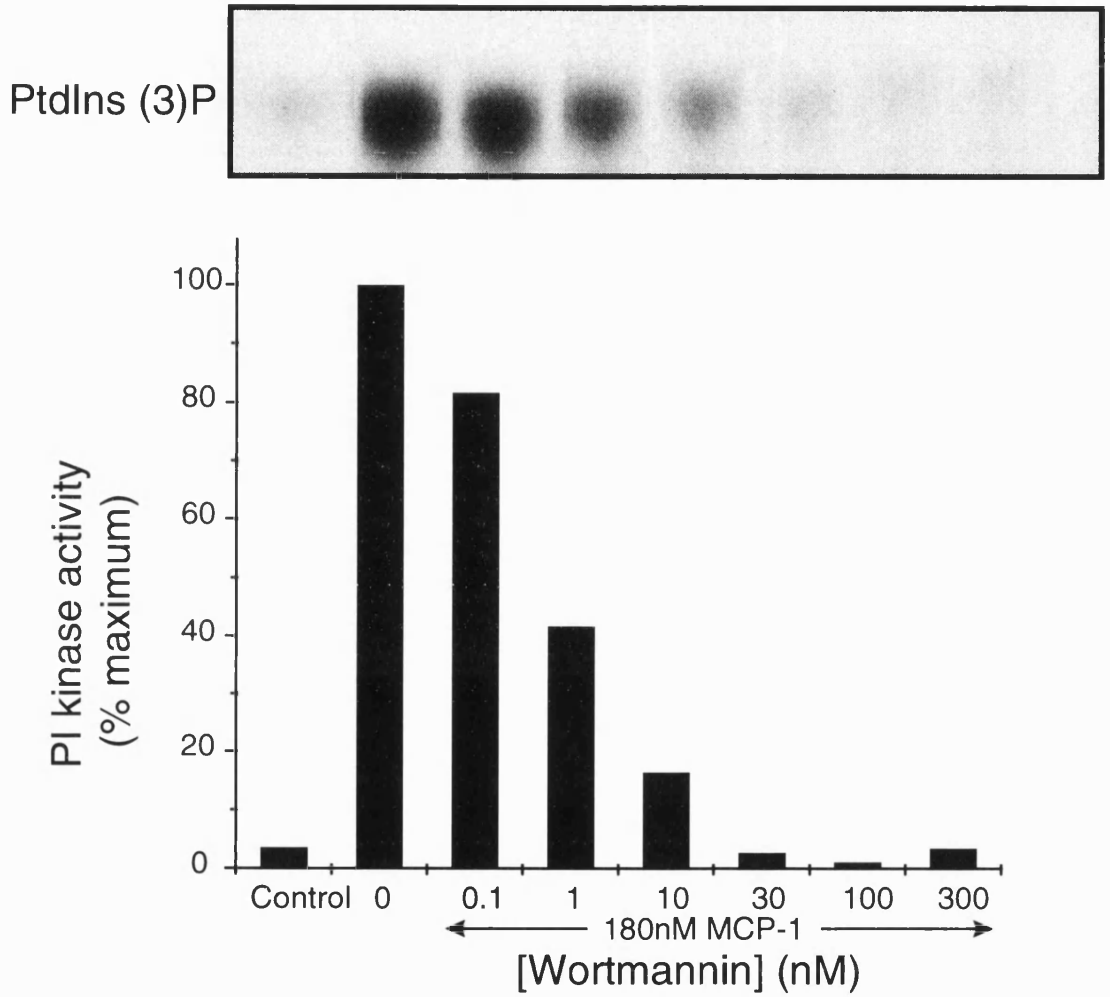


Figure 5.7 - *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells in the presence or absence of various concentrations of wortmannin. 1x10^7 cells were pre-incubated with various concentrations of wortmannin for 5 minutes and then stimulated with 180nM MCP-1 for 120 seconds. The PI 3-kinase activity was then immunoprecipitated from the cell lysates using anti-p85 coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned image of the autoradiograph as well as the densitometric analysis. The results are expressed as PI kinase activity as a percentage of the maximum. The data is representative of at least three separate experiments.

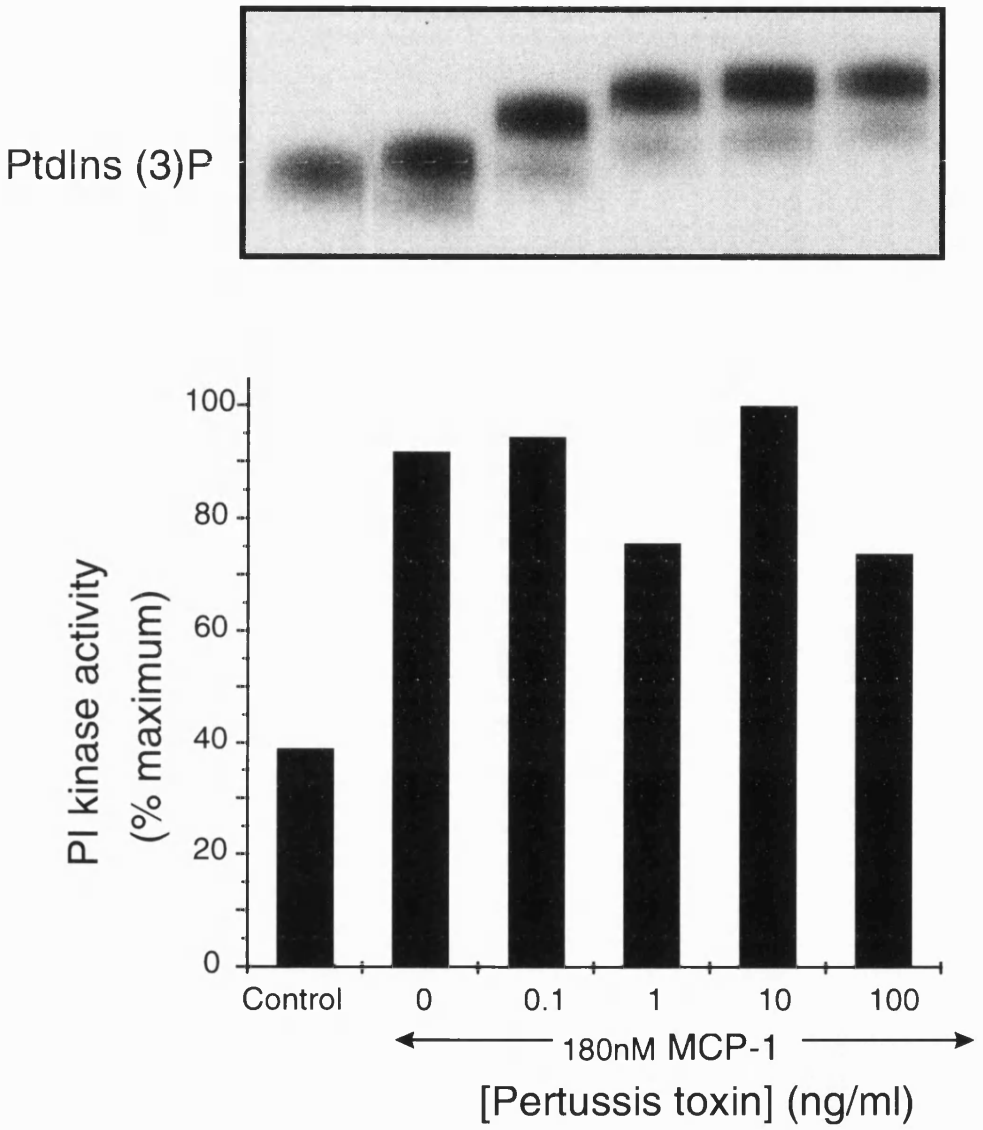


Figure 5.8 - *In vitro* lipid kinase assay of MCP-1 stimulated THP-1 cells in the presence or absence of various concentrations of pertussis toxin. The cells were pre-treated with pertussis toxin for 16 hours and then $1x10^7$ cells were stimulated with 180nM MCP-1 for 120 seconds. Cell lysates were then prepared, immunoprecipitated using anti-p85 coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned image of the autoradiograph as well as the densitometric analysis. The results are expressed as PI kinase activity as a percentage of the maximum. The data is representative of at least three separate experiments.

5.2 MCP-1-induced activation of PI 3-kinase in CC CKR 2A and 2B transfected cells

5.2.1 MCP-1-induced accumulation of D-3 phosphatidylinositol lipids

Using the 30 second time-point observed to be maximal for MCP-1-induced PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 accumulation in THP-1 cells, the effects of MCP-1 on PI 3-kinase product levels in the CC CKR 2A and 2B transfectants were examined. EGF has already been reported to induce activation of PI 3-kinase in epithelial cells and was used as a positive control (Soltoff *et al* 1994) (Carter and Downes, 1992).

PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 all demonstrated dose-dependent increases upon MCP-1 stimulation in the CC CKR 2A transfected cells (figure 5.9). The increases in PtdIns(3,4,5)P_3 demonstrated bell-shaped characteristics, with 60nM MCP-1 inducing a maximal increase. In contrast, the MCP-1-induced increases in PtdIns(3)P and PtdIns(3,4)P_2 were both maximal at 180nM MCP-1. The elevation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in these cells was very similar to those observed in the THP-1 cells following MCP-1 stimulation. In contrast, the MCP-1-induced increase in PtdIns(3)P was not detected in THP-1 cells. Stimulation with 100ng/ml EGF produced a 2-fold increase in the levels of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in these cells.

In stark contrast to the results obtained with the CC CKR 2A transfectants, the CC CKR 2B transfected cells demonstrated a very different pattern of PI 3-kinase product accumulation. A very small increase in PtdIns(3)P was observed in these cells

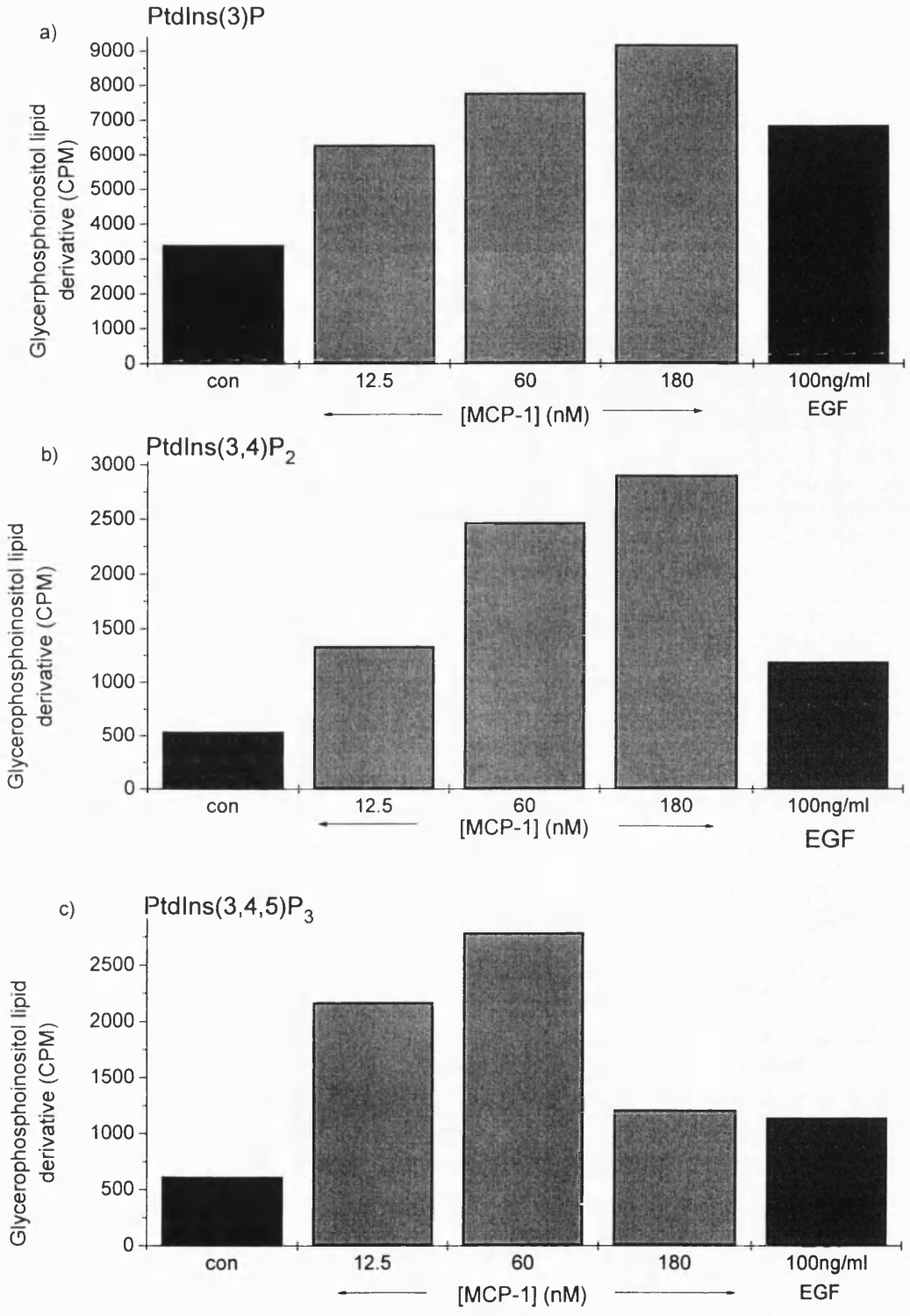


Figure 5.9 - Changes in a) PtdIns(3)P, b) PtdIns(3,4)P_2 and c) PtdIns(3,4,5)P_3 in CC CKR 2A transfected cells. 1x10^7 [^{32}P] labelled cells were stimulated with either vehicle (■), MCP-1(▩) or EGF (■) at the given concentrations for 30 seconds and then extracted, deacylated and analysed as given in section 2.2.6. The data is representative of four separate experiments.

following MCP-1 stimulation. An increase in the PtdIns(3,4)P_2 levels was also detected in response to MCP-1, with a maximal 3-fold increase following 12.5nM MCP-1 stimulation, but there was a very high basal level of PtdIns(3,4,5)P_3 present in these cells (figure 5.10). This basal level of PtdIns(3,4,5)P_3 was at least 7-fold higher than that observed in the CC CKR 2A transfected cells and was not increased upon MCP-1 stimulation and in some cases the PtdIns(3,4,5)P_3 levels were reduced by MCP-1. Stimulation of the cells with 100ng/ml EGF did not produce any increase in the levels of PtdIns(3)P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 above basal levels.

Stimulation of the untransfected cells with 100ng/ml EGF induced a 10-fold and 2-fold increase in PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively but no change in the PtdIns(3)P levels (figure 5.11). Stimulation with 12.5-180nM MCP-1 did not induce an increase in PI 3-kinase products in these untransfected cells.

5.2.2 Immunoprecipitation of MCP-1-induced PI 3-kinase in CC CKR 2A and 2B transfectants

The effects of MCP-1 on the *in vitro* lipid kinase activity in the CC CKR 2A and 2B transfected cells were investigated using the 120 second time-point optimal for the THP-1 cells.

In contrast to the marked increases in the levels of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 observed in the CC CKR 2A transfectants following MCP-1 stimulation, no increase in the immunoprecipitated PI 3-kinase activity was detected in these cells (figure 5.12). Similar results were also observed with EGF. Stimulation of the cells with 100ng/ml EGF produced no increase in the immunoprecipitated PI 3-kinase activity. If anything, the PI 3-kinase activity was reduced in the presence of EGF.

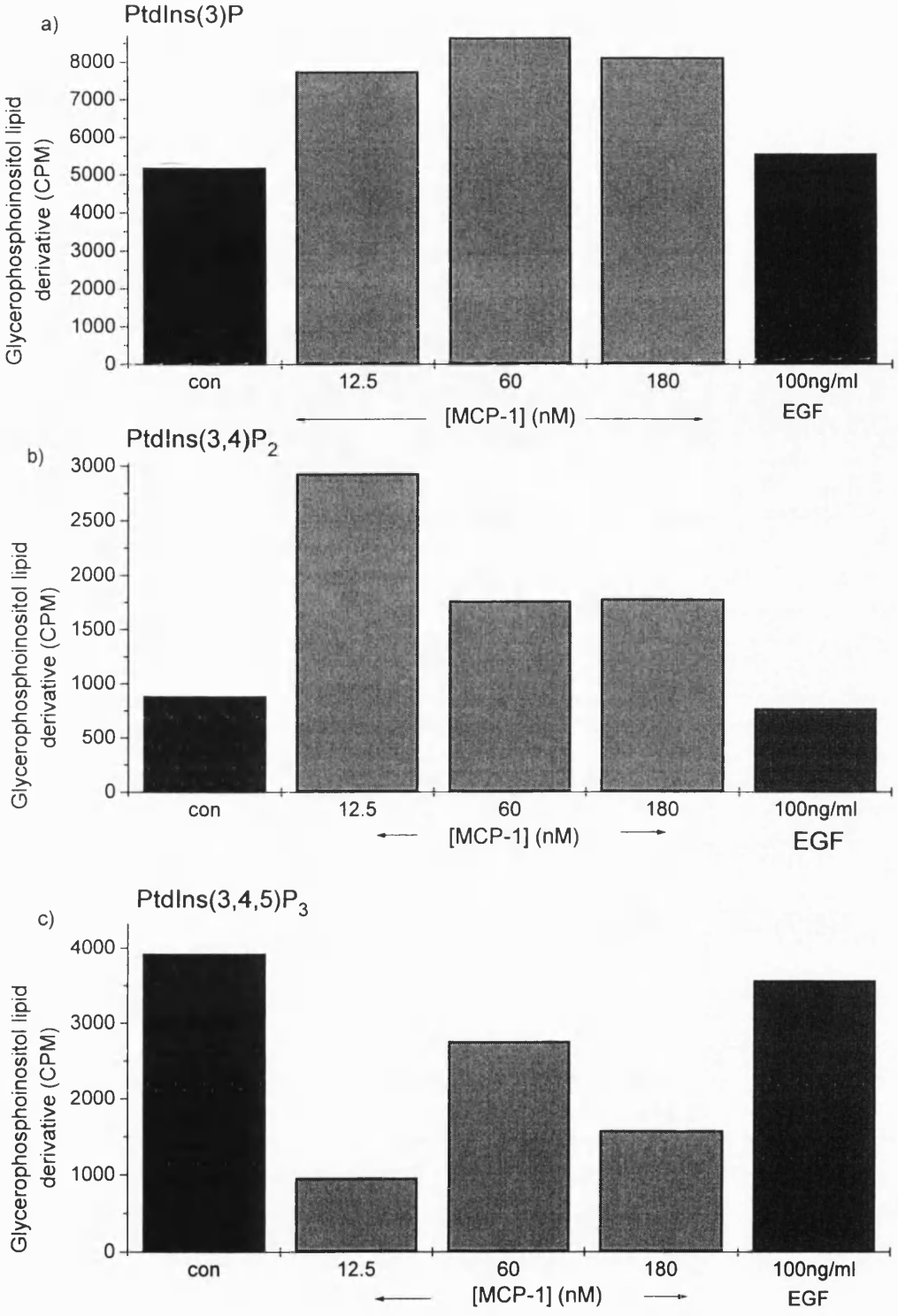


Figure 5.10 - Changes in a) PtdIns(3)P, b) PtdIns(3,4)P_2 and c) PtdIns(3,4,5)P_3 in CC CKR 2B transfected cells. 1x10^7 [^{32}P] labelled cells were stimulated with either vehicle (■), MCP-1(▩) or EGF (■) for 30 seconds at the given concentrations and then extracted, deacylated and analysed as given in section 2.2.6. The data is representative of four separate experiments.

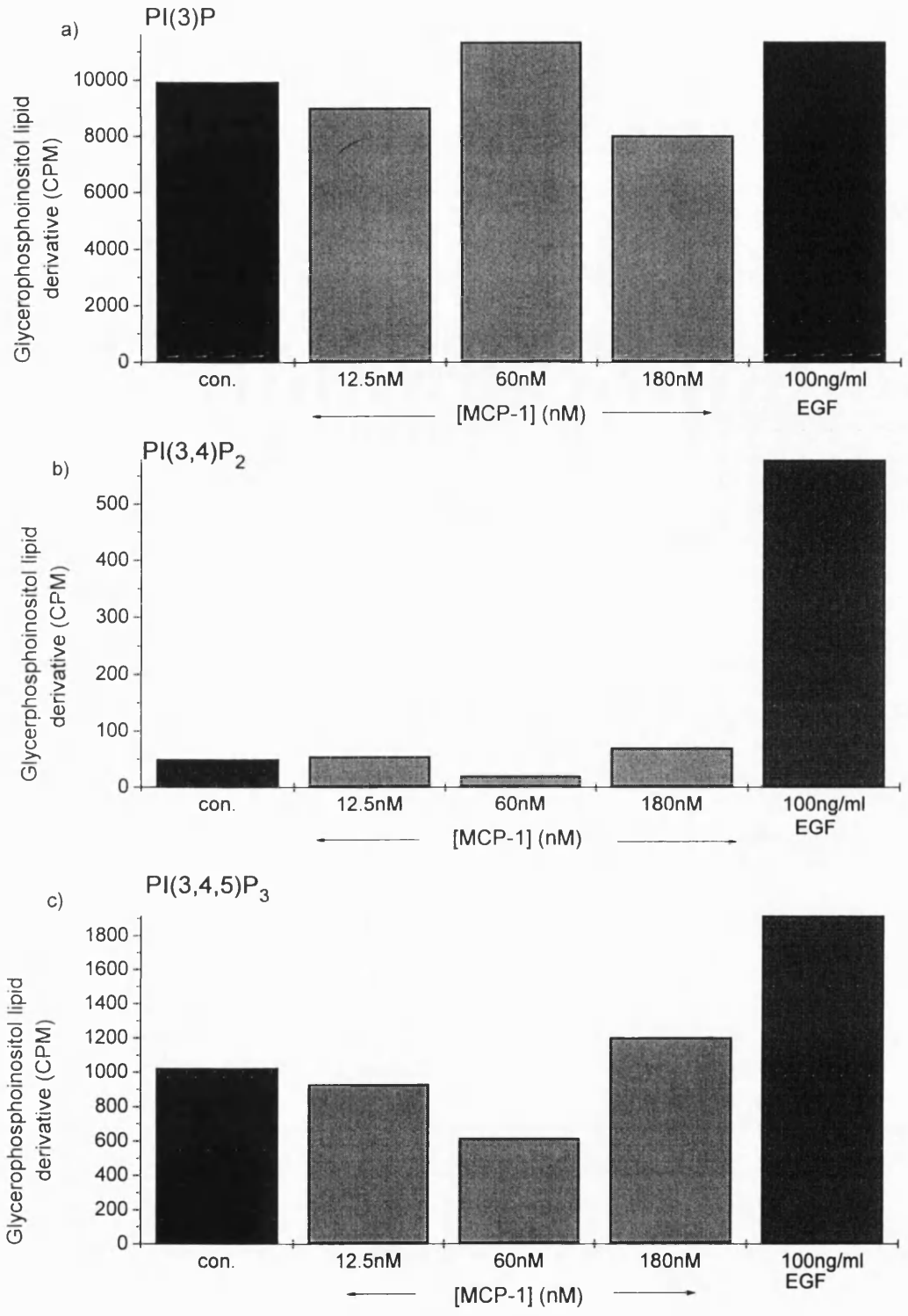


Figure 5.11 - Changes in a) PI(3)P, b) PI(3,4)P_2 and c) PI(3,4,5)P_3 in untransfected HEK 293 cells. 1x10^7 [^{32}P] labelled cells were stimulated with either vehicle (■) , MCP-1 (▒) or EGF (■) at the given concentrations for 30 seconds and then extracted, deacylated and analysed as given in section 2.2.6. The data is representative of three separate experiments.

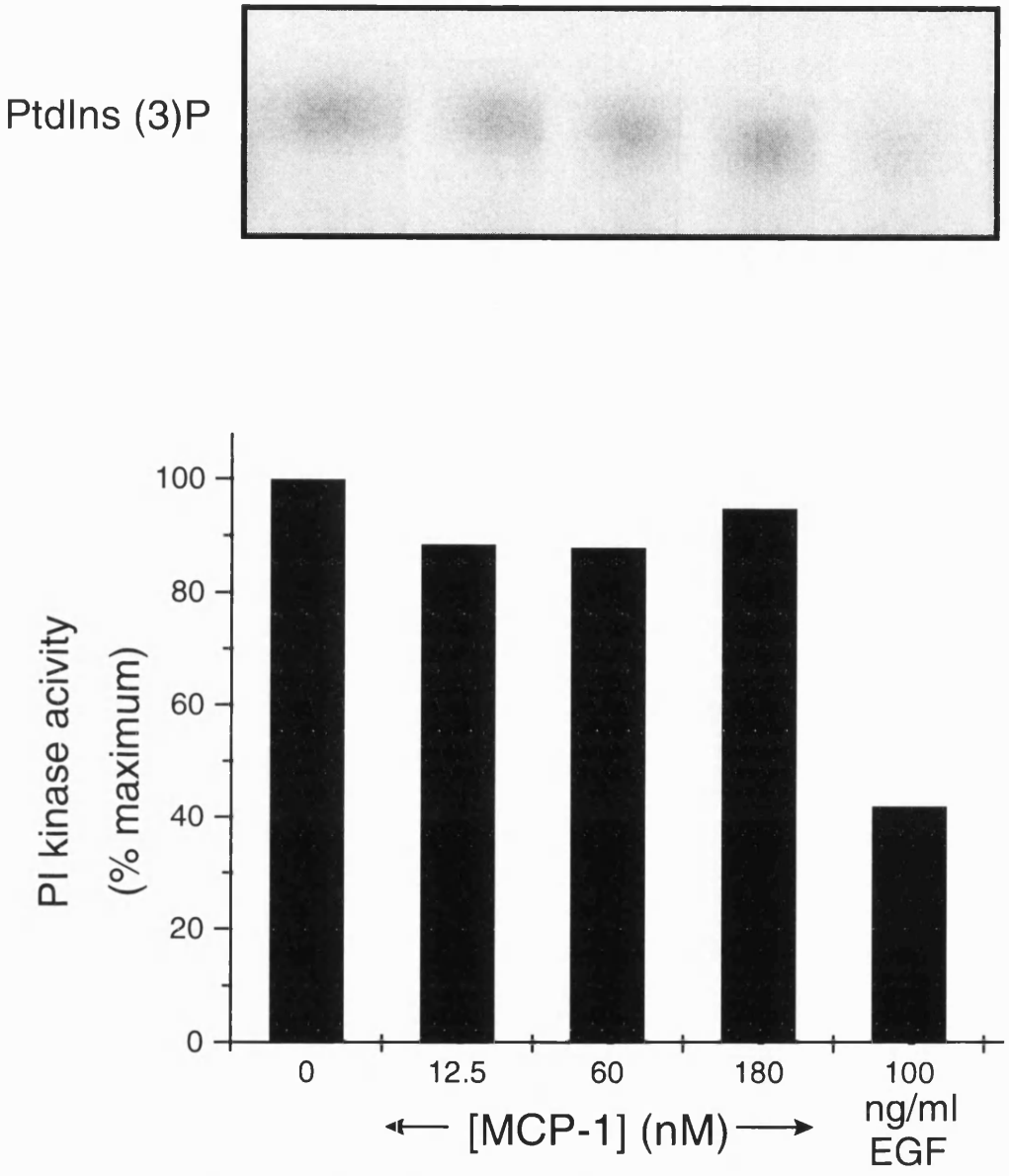


Figure 5.12 - *In vitro* lipid kinase assay of CC CKR 2A cells stimulated with various concentrations of MCP-1 or 100ng/ml EGF. $1x10^7$ cells were stimulated with various concentrations of MCP-1 or 100ng/ml EGF for 120 seconds and then the PI 3-kinase activity was immunoprecipitated from the cell lysates using anti-p85 antibody coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned image of the autoradiograph as well as the densitometric analysis expressing the results as PI kinase activity as a percentage of the maximum. The data is representative of at least three separate experiments.

Figure 5.13 shows the effects of MCP-1 and EGF on the immunoprecipitated PI 3-kinase activity from the CC CKR 2B transfected cells. Stimulation of these cells with various concentrations of MCP-1 resulted in an increase in the immunoprecipitated PI 3-kinase activity. The concentrations of MCP-1 required for the maximal activation of this immunoprecipitated PI 3-kinase activity in the CC CKR 2B transfectants were lower than required in THP-1 cells. These results in the CC CKR 2B transfectants are in contrast to the results obtained studying the accumulation of D-3 PtdIns lipids, in which there was a very high basal PtdIns(3,4,5)P_3 level which was not increased by MCP-1 stimulation. Also, stimulation of the cells with 100ng/ml EGF, which did not induce an increase in PtdIns(3)P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, induced a 4-fold increase in the immunoprecipitated PI 3-kinase activity.

5.3 Determination of protein tyrosine kinase coupling to MCP-1 receptors in THP-1 cells and CC CKR 2A and 2B transfectants

The activation of the PTK-coupled PI 3-kinase is thought to be through the interaction of tyrosine phosphorylated proteins with the SH2 domains of the p85 regulatory subunit (Carpenter *et al.* 1993). Following the identification of MCP-1-induced activation of the PTK/SH2-coupled PI 3-kinase by immunoprecipitation and *in vitro* lipid kinase assay, it was important to investigate 1) whether MCP-1 induced tyrosine phosphorylation of cellular proteins, 2) whether these tyrosine phosphorylated proteins interacted with the p85 subunit of PI 3-kinase and 3) whether PI 3-kinase was phosphorylated on tyrosine.

This was performed by activating the cells and subsequently immunoprecipitating either the tyrosine phosphorylated proteins using the anti-phosphotyrosine antibody coupled to protein G sepharose beads or immunoprecipitating the p85 subunit using

an anti-p85 antibody coupled to proteins G beads. The proteins were separated by SDS-PAGE, Western blotted and developed using the anti-phosphotyrosine antibody, 4G10.

Attempts to detect tyrosine phosphorylation in whole cell lysates were unsuccessful due to the amount of background making any potential small increases impossible to detect.

Figure 5.14a shows the three proteins which were tyrosine phosphorylated in a time-dependent manner following 180nM MCP-1 stimulation of THP-1 cells. These proteins had molecular weights of 120, 80 and 50kDa. The 80 kDa and the 50kDa proteins demonstrated similar phosphorylation kinetics. They were maximally phosphorylated at 30 seconds and the levels of phosphorylation had declined back to basal levels by 120 seconds. The 120kDa band exhibited more rapid phosphorylation kinetics than the other two proteins. Phosphorylation of the 120kDa protein was only clearly observed at 30 seconds and by 120 seconds its phosphorylation level was reduced to control.

To further characterise the protein tyrosine kinase activity stimulated by MCP-1 in THP-1 cells, both pertussis toxin and wortmannin were employed. Figure 5.14b demonstrates that the MCP-1-induced tyrosine phosphorylation was completely abrogated by the pre-incubation of the cells for 16 hours with 100ng/ml pertussis toxin. In contrast, pre-treatment of the cells for 5 minutes with 100nM wortmannin had no effect on the tyrosine phosphorylation stimulated by MCP-1.

Figure 5.15 shows preliminary data demonstrating the tyrosine phosphorylated protein which was co-precipitated with the p85 subunit of PI 3-kinase in the THP-1

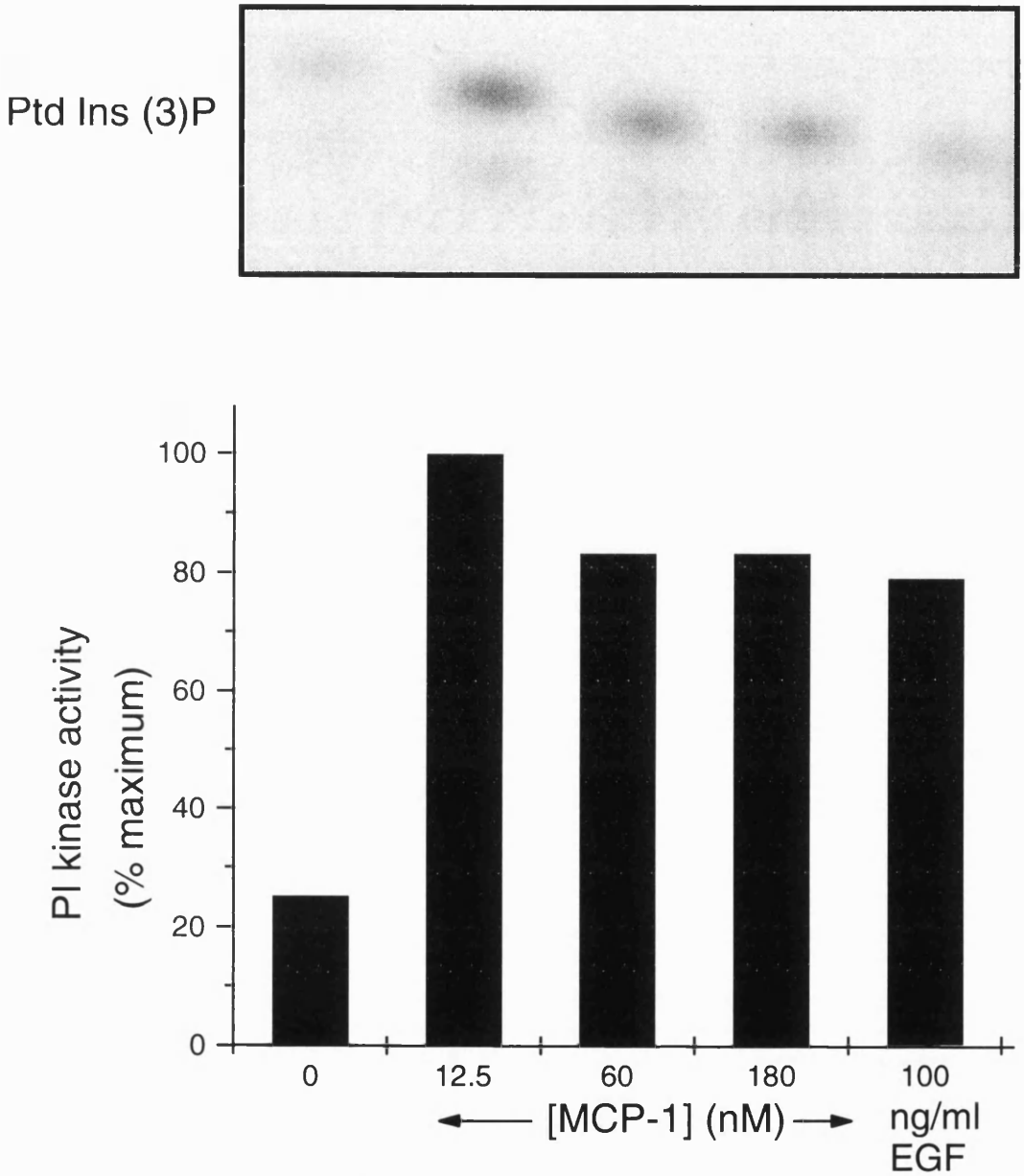


Figure 5.13 - *In vitro* lipid kinase assay of CC CKR 2B cells stimulated with various concentrations of MCP-1 or 100ng/ml EGF. $1x10^7$ cells were stimulated with various concentrations of MCP-1 or 100ng/ml EGF for 120seconds and then the PI 3-kinase activity immunoprecipitated from the cell lysates using anti-p85 antibody coupled protein G beads and subjected to an *in vitro* lipid kinase assay as given in section 2.2.6.2. This figure shows the scanned image of the autoradiograph as well as the densitometric analysis expressing the results as PI kinase activity as a percentage of the maximum. The data is representative of at least three separate experiments.

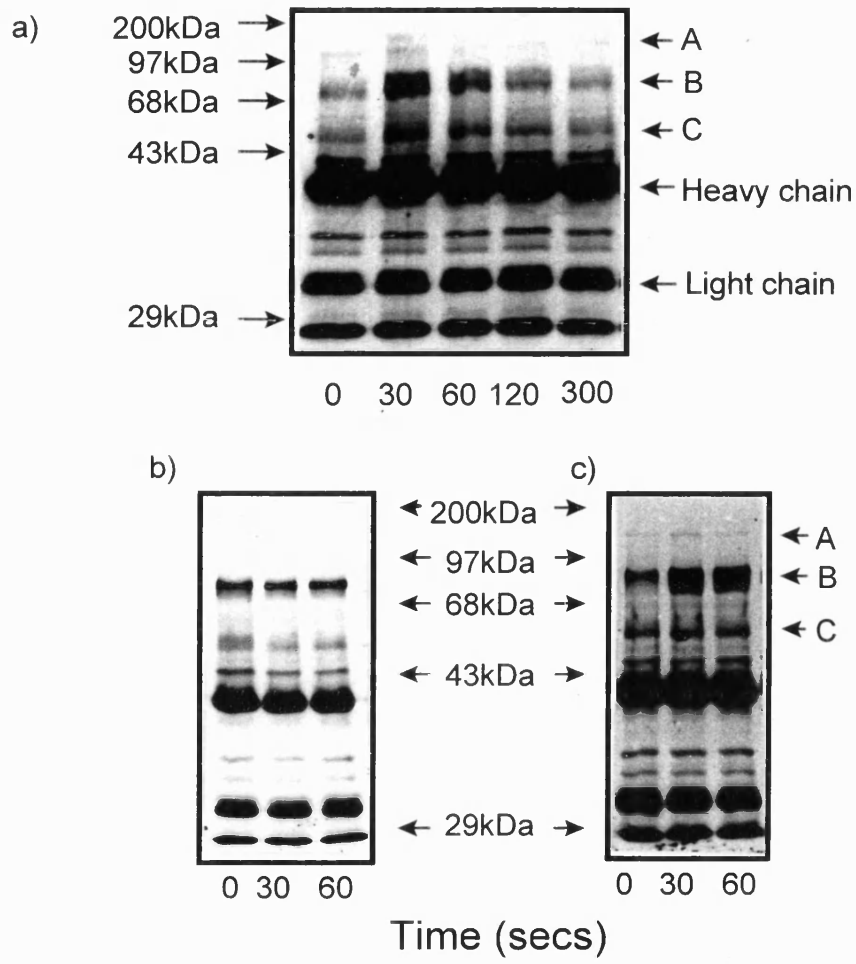


Figure 5.14 - Tyrosine phosphorylation of proteins following MCP-1 stimulation of THP-1 cells. $1x10^7$ THP-1 cells were stimulated and cell lysates prepared. These were then immunoprecipitated with PY20-coupled protein G beads and the proteins separated by SDS-PAGE, Western blotted and developed with 4G10 antibody, as given in section 2.2.7. a) This figure shows a photograph of an autoradiograph following 180nM MCP-1 stimulation for various times and is representative of three separate experiments. b) This figure shows the effects of 16 hour pre-treatment of the cells with 100ng/ml pertussis toxin and c) shows the effects of 100nM wortmannin pre-treatment for 5 mins prior to MCP-1 stimulation. These photographs are represenative of two separate experiments. The molecular weight markers and the heavy and light chains of the antibody used for immuno-precipitation are indicated by arrows. A, B and C indicate the proteins phosphorylated by MCP-1. The molecular weights are 120, 80 and 50kDa respectively.

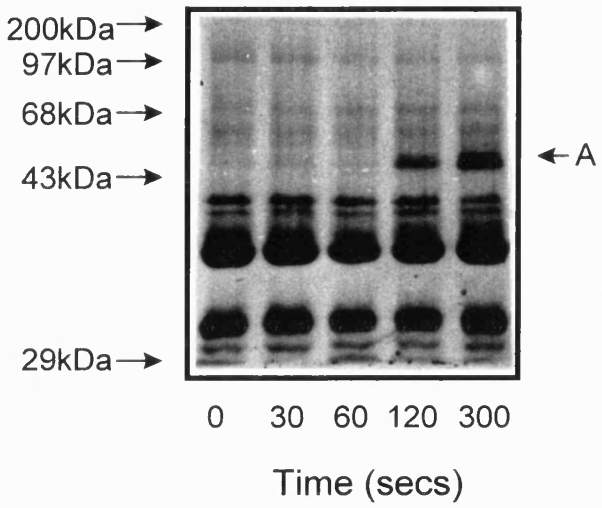


Figure 5.15 - Tyrosine phosphorylated proteins associated with the p85 subunit of PI 3-kinase in THP-1 cells. $1x10^7$ THP-1 cells were stimulated with 180nM MCP-1 for various times and then cell lysates prepared. In this experiment the lysates were incubated for 1 hour with the anti-p85 antibody at 4°C and then rotated for 1 hour with 30μl of a 50% suspension of protein G sepharose beads at 4°C. The proteins were separated by SDS-PAGE, Western blotted and developed with 4G10 antibody, as given in section 2.2.7. This figure shows a photograph of the autoradiograph and is preliminary data from one experiment. The molecular weight markers are indicated by the arrows. A indicates the phosphorylated protein associated with p85 and its molecular weight is 55kDa.

cells. This band had a molecular weight of approximately 55 kDa and its phosphorylation was delayed. The protein was only just visible at 30 and 60 seconds but was greatly increased by 120 seconds and was maximally phosphorylated by 300 seconds. This was comparable with the kinetics of the immunoprecipitated PI 3-kinase activity activated by MCP-1 in these THP-1 cells. However, the time-course of phosphorylation of this 55kDa band was different to the time-course of phosphorylation observed for the other proteins present in the anti-phosphotyrosine immunoprecipitates. There was no evidence that the p85 subunit itself was tyrosine phosphorylated.

Given the results obtained in the THP-1 cells following MCP-1 stimulation, the ability of MCP-1 to activate protein tyrosine kinase activity in the CC CKR 2A and 2B transfected cells was investigated. The preliminary data shown in figure 5.16a demonstrates that very little protein phosphorylation was detected in the CC CKR 2A transfectants following MCP-1 stimulation. The 80kDa protein, observed to be phosphorylated by MCP-1 in the THP-1 cells, demonstrated a very small increase in the level of phosphorylation at 60 seconds after MCP-1 stimulation of these CC CKR 2A transfectants. However, no other proteins were observed to be phosphorylated following MCP-1 stimulation of the CC CKR 2A transfected cells.

In contrast, preliminary data indicates that MCP-1 induced a time-dependent increase in the tyrosine phosphorylation of three proteins in the CC CKR 2B transfected cells (figure 5.16b). These phosphorylated proteins demonstrate the same molecular weights as the phosphorylated proteins detected following MCP-1 stimulation of the THP-1 cells. The 120, 80 and 50kDa proteins phosphorylated upon MCP-1 stimulation of the CC CKR 2B transfectants were maximally phosphorylated 60 seconds after MCP-1 stimulation and had declined to basal levels by 300 seconds. This time-course of phosphorylation of these proteins in the CC CKR 2B transfectants

was slightly delayed compared to the tyrosine phosphorylation observed in the THP-1 cells, which was maximal 30 seconds after MCP-1 stimulation.

5.4 Determination of potential downstream effectors of PI 3-kinase activated by MCP-1

The number of downstream targets of PI 3-kinase is now increasing very rapidly and, to date, includes PKCs ε, δ, η and ζ, PKB and p70 S6 kinase (reviewed (Ward *et al.* 1996)). One method of investigating the possible activation of p70 S6 kinase is using a so-called 'band shift' assay. The cells are stimulated and lysed and the reduced cell lysates separated on an SDS-PAGE gel. The proteins are transferred onto a nitrocellulose membrane and the p70 S6 kinase is detected by immunoblotting using a specific monoclonal antibody. This antibody recognises two isoforms of p70 S6 kinase, namely the αI and the αII isoform. In the course of activation, the enzyme becomes phosphorylation and has reduced gel mobility. Thus, the p70 S6 kinase band detected by immunoblotting appears to shift upwards. As PI 3-kinase activation has already been identified in THP-1 cells following MCP-1 stimulation, the phosphorylation of the downstream target p70 S6 kinase was investigated using the 'band shift' assay.

Figure 5.17 shows that MCP-1 induced a time-dependent increase in the number of p70 S6 kinase bands appearing in THP-1 cells. Only the αII isoform of p70 S6 kinase demonstrated any MCP-1-induced band shift. The increase in the number of bands, from three to four, was notable 2 minutes after 180nM MCP-1 stimulation and was maximal 15 minutes after stimulation. The αII band appeared to be declining to basal levels by 30 minutes.

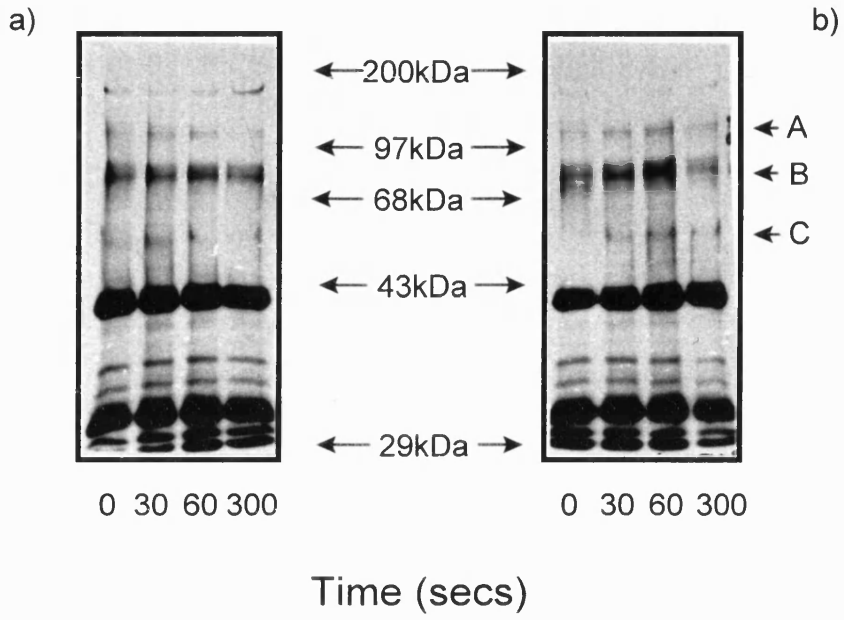


Figure 5.16 - Tyrosine phosphorylation of proteins following MCP-1 stimulation of CC CKR 2A and 2B transfected cells.
$1x10^7$ cells were stimulated and cell lysates prepared. These were then immunoprecipitated with PY20-coupled protein G beads and the proteins separated by SDS-PAGE, Western blotted and developed with 4G10 antibody, as given in section 2.2.7. a) This figure shows a photograph of an autoradiograph following stimulation of CC CKR 2A cells with 180nM MCP-1 for various times. b) This shows a photograph of an autoradiograph following stimulation of CC CKR 2B cells with 180nM MCP-1 for various times. This is preliminary data from one experiment. The molecular weight markers are indicated by the arrows. A, B and C indicate the proteins phosphorylated by MCP-1. The molecular weights are 120, 80 and 50kDa respectively.

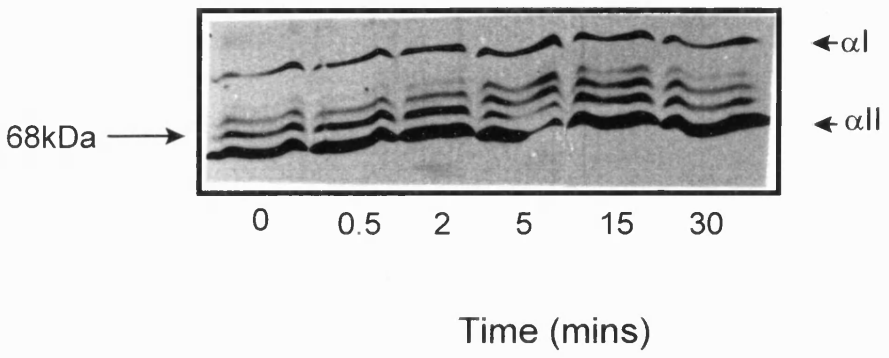


Figure 5.17 - Effects of MCP-1 on p70 S6 kinase phosphorylation in THP-1 cells. $1x10^7$ cells were stimulated with 180nM MCP-1 for various times and cell lysates prepared. The proteins were then acetone precipitated and separated using SDS-PAGE, Western blotted and developed using an anti-p70 S6 kinase antibody as given in section 2.2.8. This figure is a photograph of an auto-radiograph and is representative of three separate experiments. The molecular weight marker is indicated by the arrow. The α1 and α2 marked by the arrows are the two isoforms of p70 S6 kinase recognised by the antibody.

5.5 Role of MCP-1 in the proliferation rate of the CC CKR 2A and 2B transfected cells

During routine culture it was observed that the CC CKR 2B transfectants appeared to grow at a faster rate than the CC CKR 2A transfectants. One possible explanation was that the HEK 293 cells could be producing MCP-1 that was acting on the receptors transfected into these cells and inducing differences in growth rates. The variation in MCP-1 receptor expression could explain the differences between the two transfectants. Also, the very high basal levels of PtdIns(3,4,5)P_3 may play an important role in these growth differences, as PtdIns(3,4,5)P_3 has already been linked to cell growth and differentiation (Coughlin *et al.* 1989). Thus, the incorporation of [^{3}H]-thymidine into these cells was investigated in the presence of increasing concentrations of FCS. The role of MCP-1 in these incorporation experiments was also investigated.

In the presence of increasing concentrations of FCS, [^{3}H]-thymidine incorporation in the CC CKR 2B transfected cells was greater than that in the CC CKR 2A transfected cells (figure 5.18a). In the presence of 1% FCS, there was a 3-fold difference between the CC CKR 2A and 2B transfectants. Interestingly, the untransfected cells demonstrated a much more rapid rate of [^{3}H]-thymidine incorporation than both of the transfectants (figure 5.18b).

The effects of MCP-1 on the rate of [^{3}H]-thymidine incorporation into the CC CKR 2A, CC CKR 2B and untransfected cells were also investigated. The cells were grown in the presence of 1% FCS with increasing concentrations of MCP-1 (1.25-60nM). 60nM MCP-1 was also pre-incubated with a neutralising anti-MCP-1 antibody prior to addition to the cells.

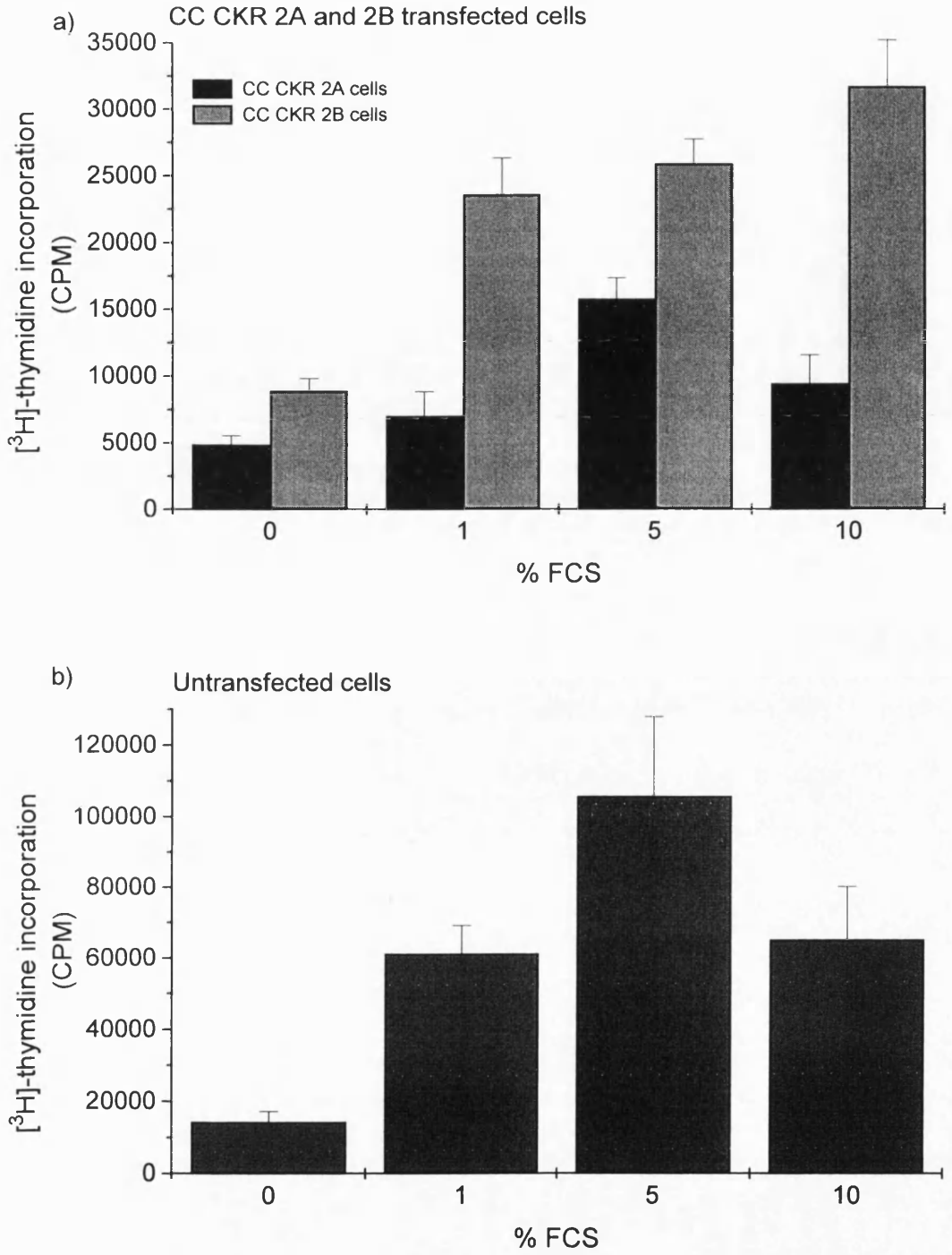


Figure 5.18 - [^{3}H]-thymidine incorporation into a) CC CKR 2A transfectants (■) and CC CKR 2B transfectants (▒) and b) untransfected HEK cells.
$5x10^3$ cells/well were plated out into 96 well plates and after 40 hours incubation they were depleted of serum for 4 hours and then grown in the presence of increasing concentrations of serum for 44 hours as given in section 2.2.10. The cells were pulsed with [^{3}H]-thymidine for the final 18 hours and then harvested and counted. The results are expressed as the mean +/- s.d. of from one experiment representative of three other separate experiments.

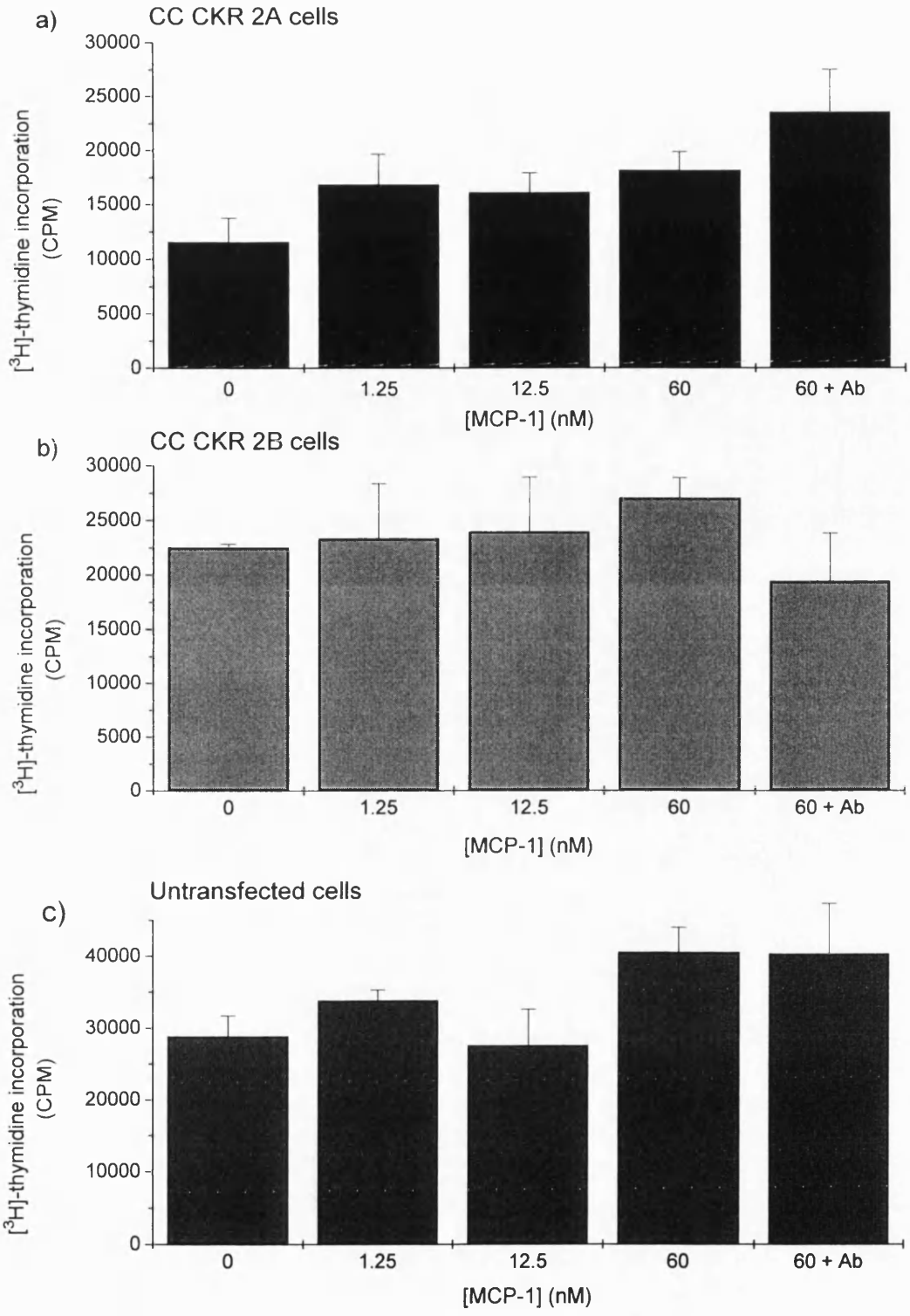


Figure 5.19 - [^{3}H]-thymidine incorporation into a) CC CKR 2A transfectants b) CC CKR 2B transfectants and c) untransfected HEK cells in response to MCP-1. 5x10^3 cells/well were plated out into 96 well plates and after 40 hours incubation they were depleted of serum for 4 hours and then grown in the presence of 1% FCS plus various concentrations of MCP-1 or 60nM MCP-1 plus a neutralising monoclonal MCP-1 antibody. The cells were pulsed with [^{3}H]-thymidine for the final 18 hours and then harvested and counted as given in section 2.2.10. The results are expressed as the mean +/-s.d. of one experiment representative of three other separate experiments.

Figure 5.19 demonstrates that there was no significant difference in the [^{3}H]-thymidine incorporation in either the CC CKR 2A or 2B transfectants in the presence of MCP-1 or the MCP-1/anti-MCP-1 antibody complex. No significant difference in [^{3}H]-thymidine incorporation was observed in the untransfected cells following MCP-1 stimulation either.

5.6 Summary

1. MCP-1 induced the accumulation of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 but not PtdIns(3)P in THP-1 cells in both a dose- and time-dependent manner. The accumulation of PtdIns(3,4,5)P_3 was almost completely inhibited by 100ng/ml pertussis toxin but was insensitive to wortmannin pre-treatment.
2. MCP-1 also activated the PTK/SH2-coupled PI 3-kinase, specifically immunoprecipitated using anti-p85 antibody coupled protein G beads, in THP-1 cells. This activation was also both dose- and time-dependent. 100nM wortmannin completely abolished the immunoprecipitated PI 3-kinase activity activated by MCP-1. In contrast, pertussis toxin pre-treatment had no effect.
3. The CC CKR 2A transfectants demonstrated an MCP-1-induced increase in the levels of PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. EGF, used as a positive control for these epithelial cells, also induced an increase in these PI 3-kinase products. In contrast, the CC CKR 2B transfected cells demonstrated very high basal levels of PtdIns(3,4,5)P_3 which were not increased by MCP-1 or EGF stimulation.
4. Immunoprecipitation of the PTK/SH2-coupled PI 3-kinase from MCP-1 stimulated CC CKR 2A cells revealed that MCP-1 did not increase the PTK/SH2-coupled PI 3-kinase activity in these cells. The positive control, EGF, also induced no increase in PI 3-kinase activity in these immunoprecipitates. In contrast, CC CKR 2B transfectants exhibited an MCP-1-induced dose-dependent increase in the

PTK/SH2-coupled PI 3-kinase activity present in the immunoprecipitates. EGF also produced a 4-fold increase in the immunoprecipitated PI 3-kinase activity.

5. Investigation into the activation of protein tyrosine kinases following MCP-1 stimulation demonstrated that three proteins, of molecular weights 120, 80 and 50kDa, were phosphorylated in a time-dependent manner in THP-1 cells. The maximal phosphorylation time was 30 seconds. The phosphorylation of these proteins was inhibited by 100ng/ml pertussis toxin pre-treatment but was unaffected by wortmannin.
6. Immunoprecipitation of the p85 subunit of PI 3-kinase from THP-1 cells and determination of the phosphorylated proteins which co-precipitated with this subunit identified a single protein of approximately 55kDa. The phosphorylation kinetics of this protein were delayed with notable phosphorylation only occurring from 120 seconds post MCP-1 stimulation. These kinetics were similar to the kinetics observed with the immunoprecipitated PTK/SH2-coupled PI 3-kinase activation.
7. No notable protein tyrosine phosphorylation was detected in the CC CKR 2A transfected cells following MCP-1 stimulation. In contrast, proteins of molecular weight 120, 80 and 50 kDa were phosphorylated in a time-dependent manner in the CC CKR 2B transfectants upon MCP-1 stimulation.
8. MCP-1 appeared to induce a time-dependent phosphorylation of the potential downstream effector of PI 3-kinase, p70 S6 kinase, as demonstrated by the increase in the number of bands detected in the so-called 'band shift' assay. This phosphorylation was maximal at 15 minutes and returning to basal levels by 30 minutes.
9. There was a marked difference between the [^{3}H]-thymidine incorporation in the CC CKR 2A and 2B transfectants but MCP-1 did not affect this incorporation in either CC CKR 2A, CC CKR 2B or untransfected cells.

SECTION 6 : FUNCTIONAL RESPONSES ACTIVATED BY MCP-1 IN HUMAN MONOCYTES AND MONOCYTIC CELL LINES

In order to try and investigate the potential roles of the signalling pathways, demonstrated in this study to be activated by MCP-1, a number of functional responses were investigated in both human monocytes and THP-1 cells. These included chemotaxis, adhesion molecule upregulation, superoxide release and low affinity IgE receptor (CD23) upregulation.

6.1 MCP-1-induced chemotaxis in human monocytes and THP-1 cells

The movement of monocytes down a chemotactic gradient can be monitored *in vitro* using a Boyden chamber in which MCP-1 is placed in the lower well and the cells in the upper well. The two chambers are separated by a membrane with pores of diameter 5 or 8μm. The number of cells which have moved towards the stimulus is identified by staining and counting the cells on the lower side of the membrane (Falk *et al.* 1980).

Human monocytes demonstrated a dose-dependent chemotactic response to MCP-1 (figure 6.1a). The EC_{50} for this response was around 1nM. THP-1 cells also demonstrated a dose-dependent chemotactic response to MCP-1 (figure 6.1b). The EC_{50} for these cells was between 0.1 and 1nM, slightly lower than observed for human monocytes. Due to the donor variation of the monocytes and the small responses demonstrated by the THP-1 cells the effect of various inhibitors was not examined.

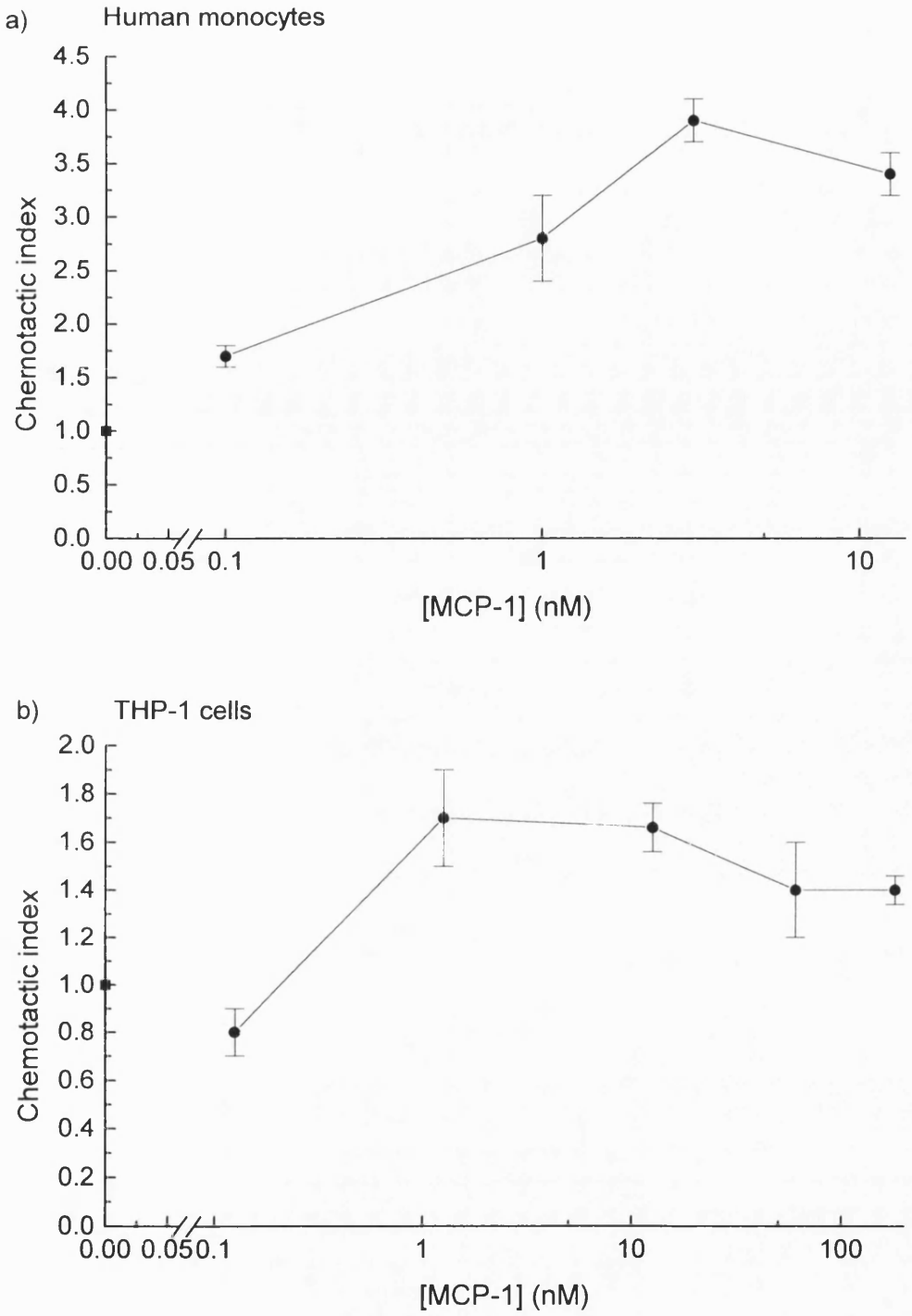


Figure 6.1 - Chemotactic response to MCP-1 in human blood derived monocytes and THP-1 cells. a) Monocytes were purified as given in section 2.2.1.3. Various concentrations of MCP-1 were placed in the lower wells and $7x10^4$ monocytes were placed in the upper wells of the Boyden chamber. The two chambers were separated by an 8μm pore size filter and incubated at 37°C for 90 minutes. b) $1.6x10^5$ THP-1 cells were placed in the upper wells separated from various concentrations of MCP-1 in the lower wells by a 5μm pore size filter and incubated at 37°C for 120 minutes. The filters were stained with Diff-Quick stain and the average number of cells from five high power fields of view calculated. The results are expressed as the mean chemotactic index from one experiment representative of six separate experiments (data +/- s.d.) for the monocytes and the mean chemotactic index from six separate experiments (mean +/- s.e.m.) for the THP-1 cells.

6.2 Upregulation of various adhesion molecules on human monocytes following MCP-1 stimulation

Adhesion molecule upregulation is important for monocyte transmigration and these cell surface molecules can be labelled with a fluoresceinated antibody and detected using the FACS. The upregulation of CD11b/CD18, CD11c/CD18 and VLA-4, upon MCP-1 stimulation was investigated in human blood derived monocytes. Although MCP-1 has already been demonstrated to induce adhesion molecule upregulation (Jiang *et al.* 1992), the signalling pathways involved in this upregulation have not been well characterised.

Following the recent publications on MCP-1-induced adhesion molecule upregulation, a 30 minute timepoint was chosen (Jiang *et al.* 1992)(Vaddi & Newton, 1994). Also, due to the upregulation of adhesion molecules upon monocyte purification, whole blood was incubated with MCP-1 and the CD14 positive monocyte population was identified in the whole blood preparation during the FACS analysis.

Figure 6.2a shows the dose-dependent increase in CD11b expression following MCP-1 stimulation, demonstrated by the increase in the relative fluorescence intensity. Maximum CD11b expression above basal levels was observed following 60nM MCP-1 stimulation. Stimulation by 1.25nM MCP-1 produced CD11b expression not markedly above unstimulated CD11b expression. The lack of fluorescence demonstrated by the IgG control eliminates any increases in the fluorescence being due to non-specific antibody binding.

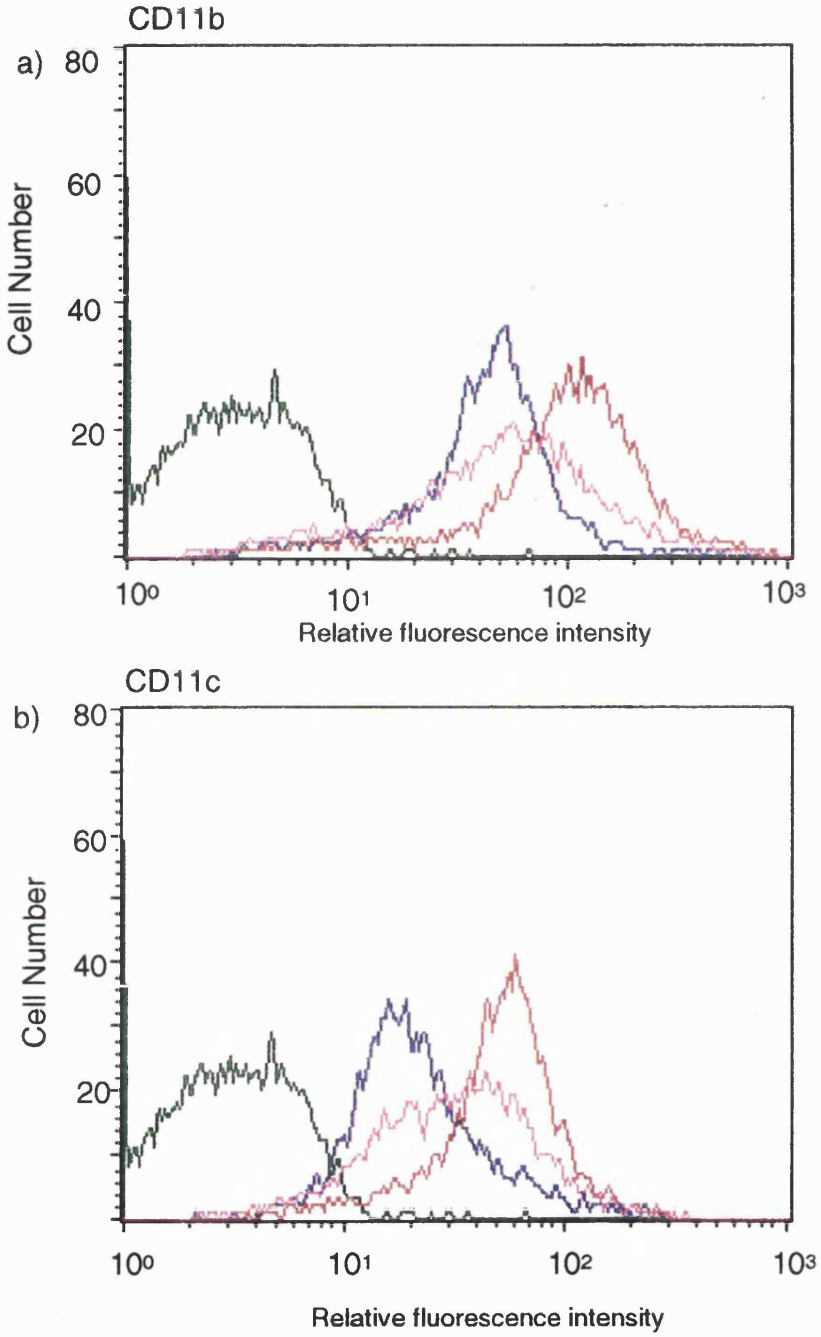


Figure 6.2 - Effects of MCP-1 on a) CD11b and b) CD11c expression on human monocytes. 100µl of whole blood was stimulated with various concentrations of MCP-1 for 30 minutes and then labelled with anti-CD11b- or CD11c-FITC antibodies as given in section 2.2.12. The data is representative of four separate experiments and is shown as the change in fluorescence after 60nM MCP-1 (——) or 1.25nM (——) compared to unstimulated (——) or IgG control (——).

Similar dose-dependent effects were observed upon investigation of the MCP-1-induced CD11c upregulation. Figure 6.2b shows that 60nM MCP-1 induced a maximal upregulation of CD11c above basal levels. 1.25nM MCP-1 did not induce any notable CD11c upregulation compared to control. Again, the IgG control was very low. Interestingly, the basal level of CD11b expression was higher than the basal CD11c expression.

VLA-4 was not upregulated on human monocytes in response to 60nM MCP-1 stimulation for 30 minutes, as demonstrated by figure 6.3 (n=3).

To further characterise the MCP-1-induced monocyte adhesion molecule upregulation the effects of wortmannin and pertussis toxin were studied on the MCP-1-induced CD11b and CD11c upregulation.

Figure 6.4 shows the effects of 100nM wortmannin pre-treatment on the upregulation of CD11b and CD11c. Preliminary data indicates that wortmannin had no marked effect on the upregulation of either CD11b or CD11c following MCP-1 stimulation. Unfortunately, pre-treatment of the whole blood for 2 hours with 100ng/ml pertussis toxin induced activation of the monocytes and thus, a very high basal expression of both CD11b and CD11c. This made it was impossible to study the effects of MCP-1 on these adhesion molecules after pertussis toxin pre-treatment.

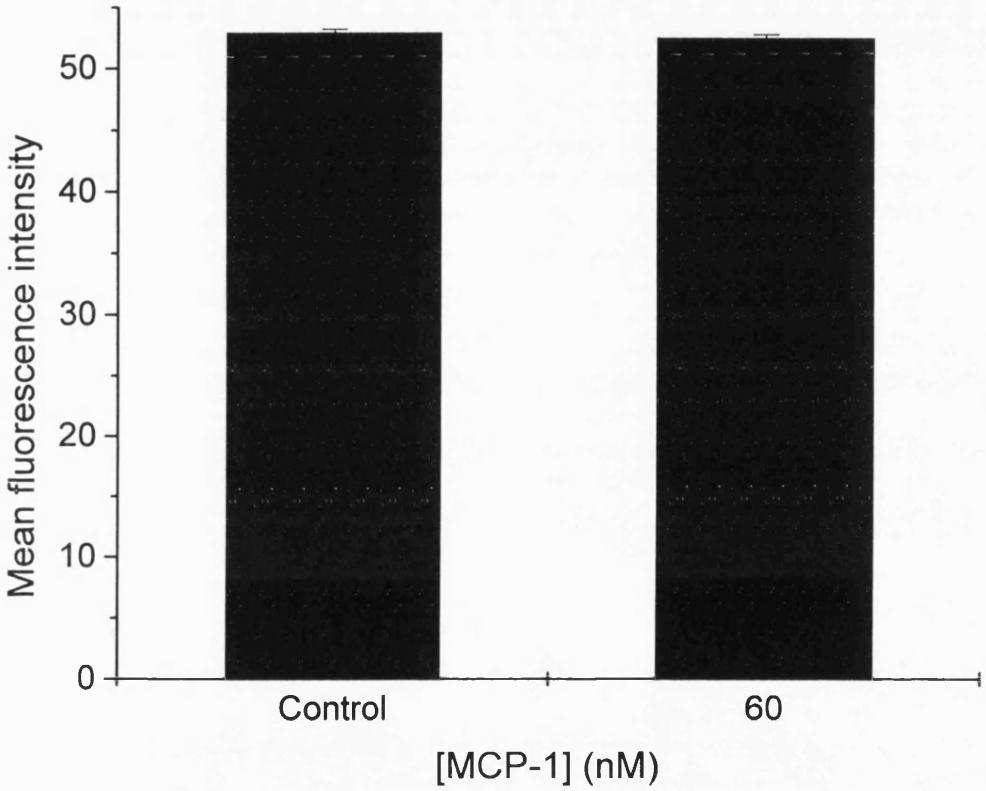


Figure 6.3 - Effects of MCP-1 on VLA-4 expression on human monocytes. 100μl of whole blood was stimulated with 60nM MCP-1 for 30 minutes and then labelled with a VLA-4-FITC antibody as given in section 2.2.12. The samples were then analysed using a FACScan. The results are expressed as the mean fluorescence intensity and are representative of three separate experiments (data +/- s.d.)

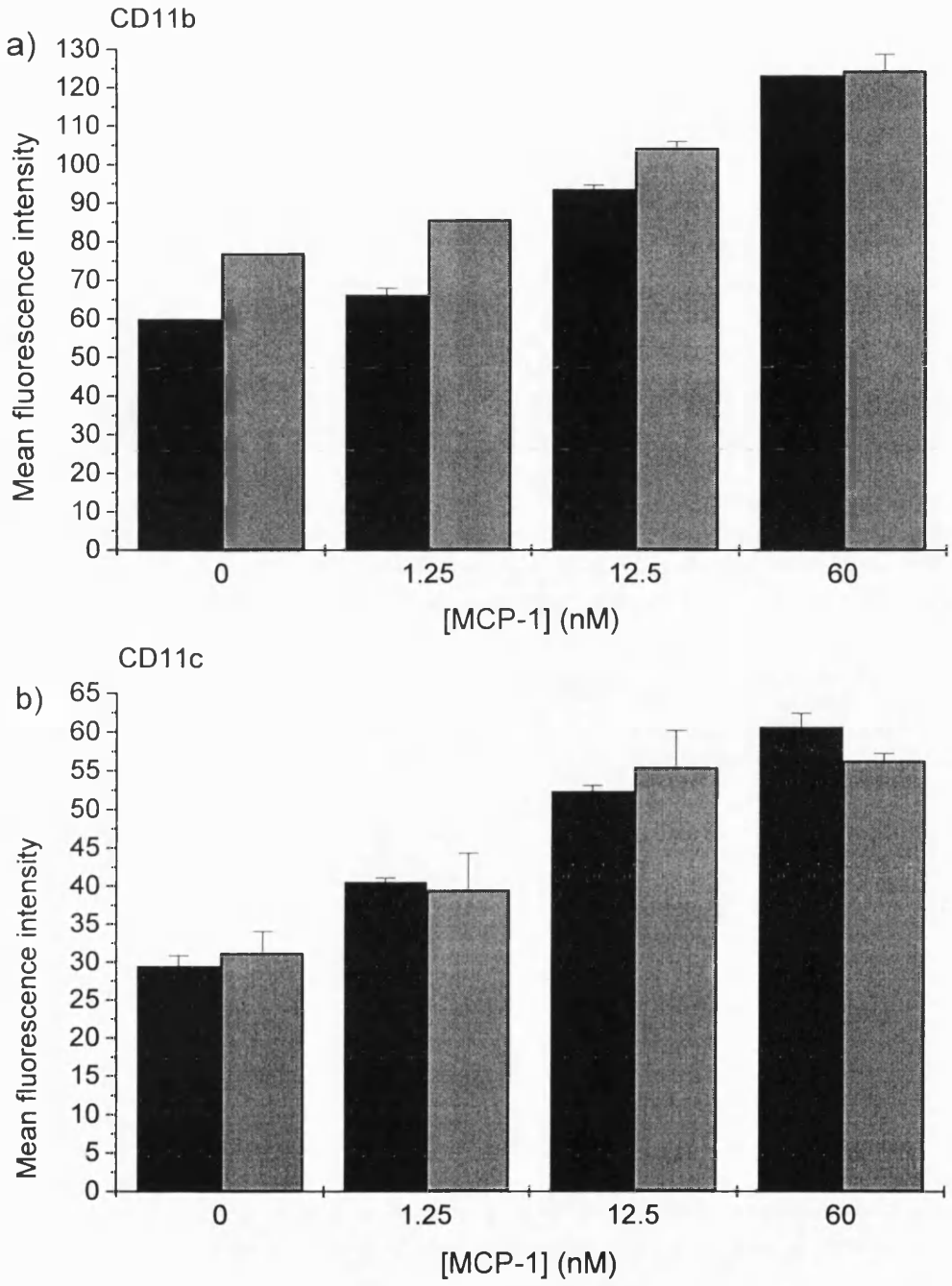


Figure 6.4 - Upregulation of a) CD11b and b) CD11c by MCP-1 on human monocytes in the absence(■) or presence (▒) of 100 nM wortmannin. Whole blood was pre-treated with 100nM wortmannin or vehicle control for 5 minutes at 37°C prior to stimulation of 100μl aliquots with various concentrations of MCP-1 for 30 minutes at 37°C. The cells were then labelled with either anti-CD11b-FITC or anti-CD11c-FITC antibodies as given in section 2.2.12. The results are preliminary data from one experiment (mean +/- s.d.). Where error bars are not visible they are smaller than the lines.

6.3 Superoxide generation in response to MCP-1 stimulation of human monocytes

One of the functional responses of human monocytes to agonist stimulation is superoxide release. There are many ways in which the generation of superoxide can be measured from cells (Jones & Hancock, 1994). Two of these methods are the oxidation of scopoletin and the reduction of ferricytochrome C. The first method measures the oxidation of scopoletin to a non-fluorescent product by H_2O_2, which is the product of the spontaneous breakdown of superoxide. The second method is a more direct method which involves the reduction of ferricytochrome C by the superoxide produced by the cells. Both of these methods were used to look at the production of superoxide from purified human blood derived monocytes in response to MCP-1 as well as C5a, fMLP and PMA as positive controls.

Figure 6.5 shows the generation of H_2O_2 in purified human monocytes following MCP-1, C5a, fMLP and PMA stimulation. Although the positive controls, C5a, fMLP and PMA all produced dose-dependent increases in H_2O_2 concentrations, MCP-1 did not induce any notable increases in H_2O_2 concentrations at any of the concentrations tested.

Due to the lack of H_2O_2 detected following MCP-1 stimulation of human monocytes using the scopoletin assay the more direct reduction of ferricytochrome C was monitored. Figure 6.6a demonstrates that MCP-1 did induce a dose-dependent increase in superoxide generation in these purified monocytes. The MCP-1-induced response was similar to that observed with C5a but smaller than that induced by fMLP and PMA.

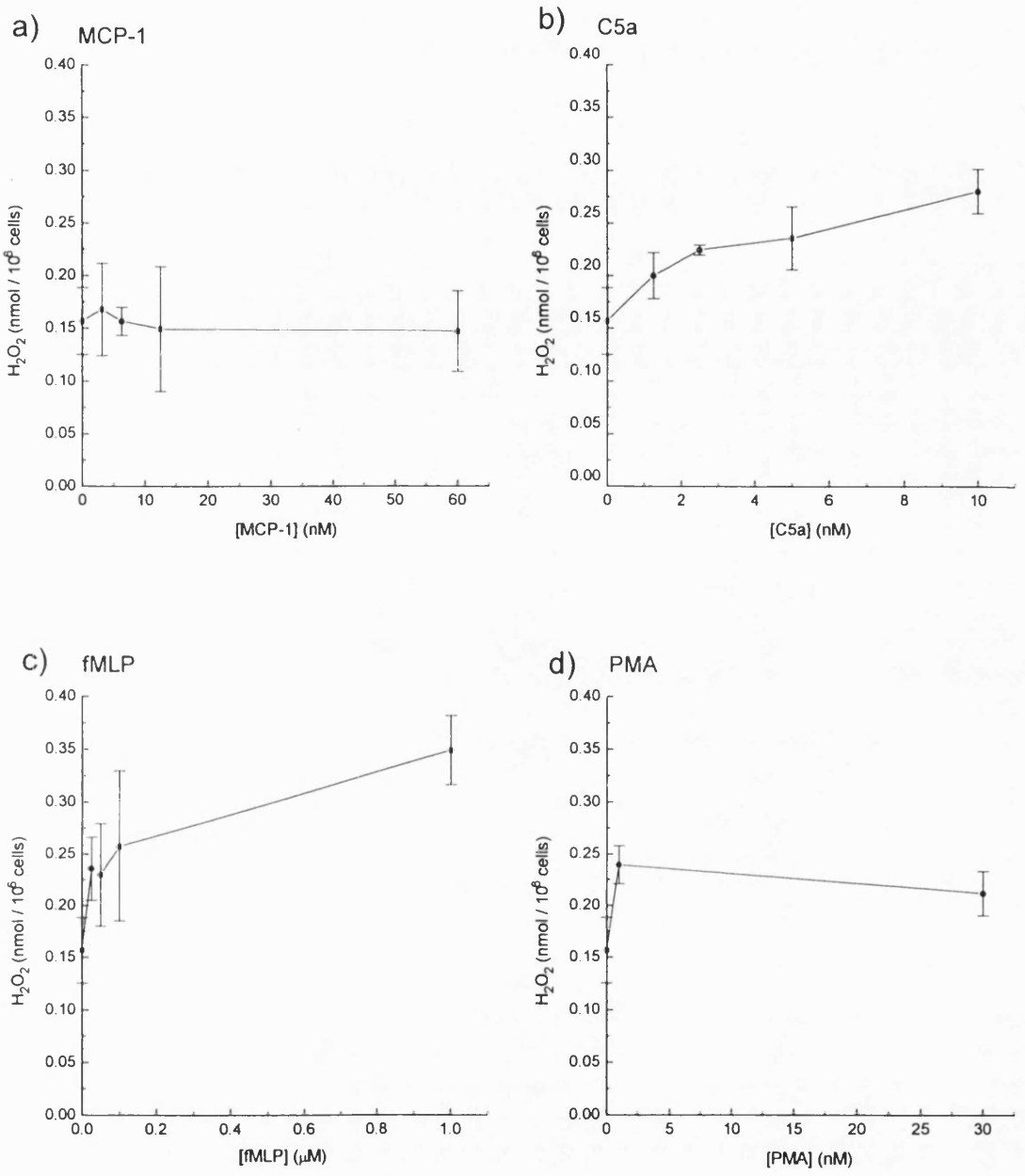


Figure 6.5 - Generation of hydrogen peroxide in purified human monocytes following stimulation by a) MCP-1, b) C5a, c) fMLP and d) PMA. The monocytes were purified as given in section 2.2.1.3. $2x10^5$ cells/well were stimulated with various concentrations of the given agonists and the reaction started as given in section 2.2.13.1. The fluorescence readings were then taken using a fluorescent plate reader and the results calculated from the standard curve. The results are expressed as mean +/- s.d. from the readings taken at 120 minutes. The data is from one experiment representative of four other separate experiments.

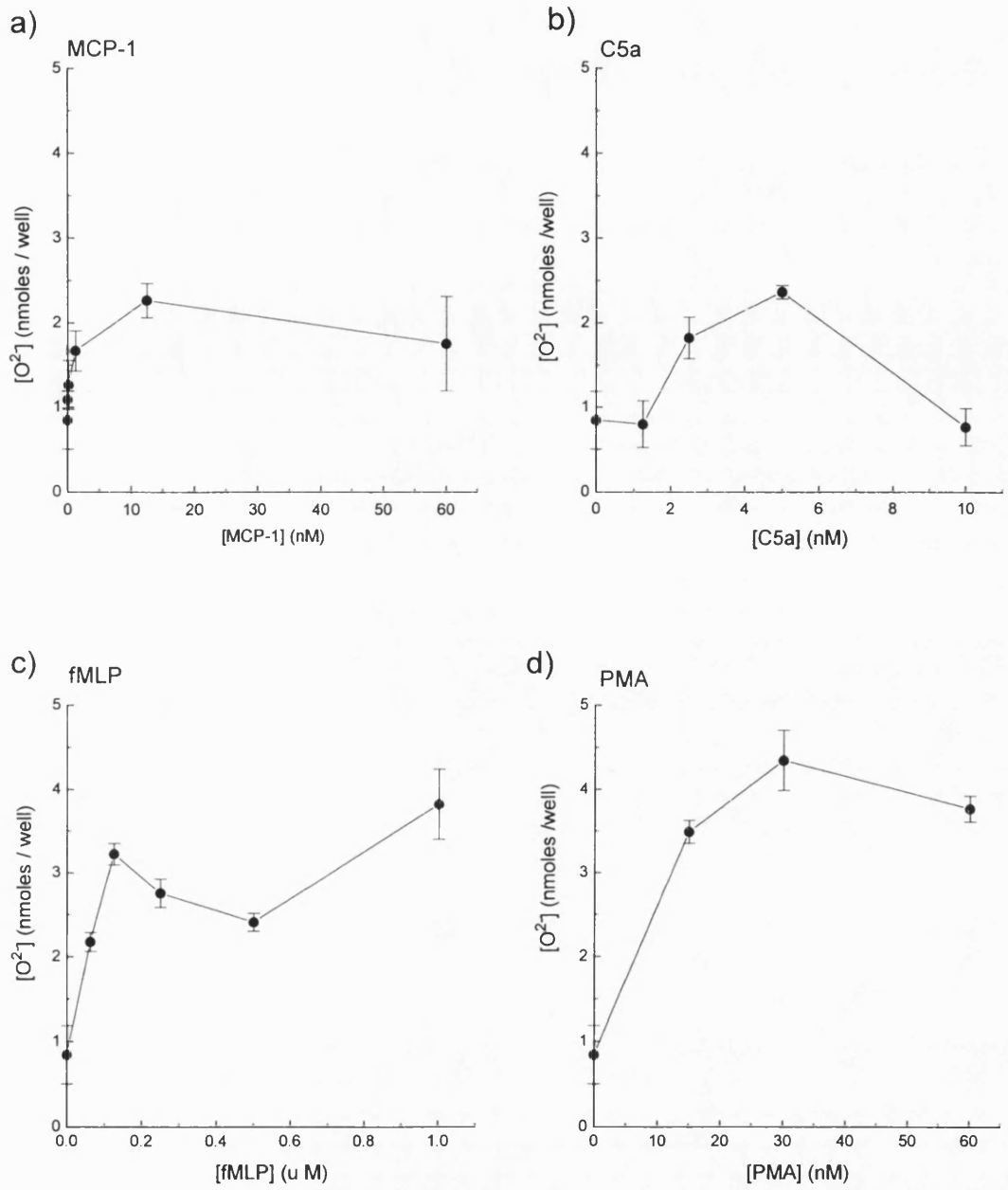


Figure 6.6 - Generation of superoxide by purified human monocytes following stimulation by a) MCP-1, b) C5a, c) fMLP and d) PMA. $1x10^6$ PBMC's well were plated out into 96 well plates and after 90 minutes incubation the non-adherent cells were washed off. The adherent monocytes were then stimulated with various concentrations of the given agonists and the reaction started as given in section 2.2.13.2. The O.D. readings were taken using a plate reader and converted into $[O^{2-}]$ using the extinction coefficient for reduced ferricytochrome C. The results are expressed as mean +/- s.d. from the readings taken at 120 minutes. The data is expressed as $[O^{2-}]$ (nmole/well) as the amount of protein per well did not vary significantly from 3mg/ml. The data is from one experiment representative of four other separate experiments.

6.4 Low affinity IgE receptor expression on human monocytes following MCP-1 stimulation

Following a number of reports of CD23 upregulation on monocytes and monocytic cell lines (Gessl *et al.* 1993)(Ouaaz *et al.* 1993), the effects of its cleavage product soluble CD23 (Paul-Eugene *et al.* 1993) as well as its increased expression on monocytes of asthmatic patients (Williams *et al.* 1992)(Demoly *et al.* 1994), the effects of MCP-1 on monocyte CD23 expression was studied. A fluoresceinated CD23 antibody was used to detect cell surface expressed CD23 using the FACS. As a positive control, IL-3 was administered in parallel as it has already been identified as a potent inducer of CD23 upregulation (Alderson *et al.* 1992).

Figure 6.7 shows that the percentage of unstimulated cells expressing CD23 was 10.72 ± 2.99% (n=3). Upon stimulation of these cells with 100ng/ml IL-3, the percentage positive cells increased to 44.64 ± 0.93% (n=3). In contrast, stimulation of these cells with 12.5nM MCP-1 did not increase the percentage of cells expressing CD23 (5.68 ± 0.77, n=3).

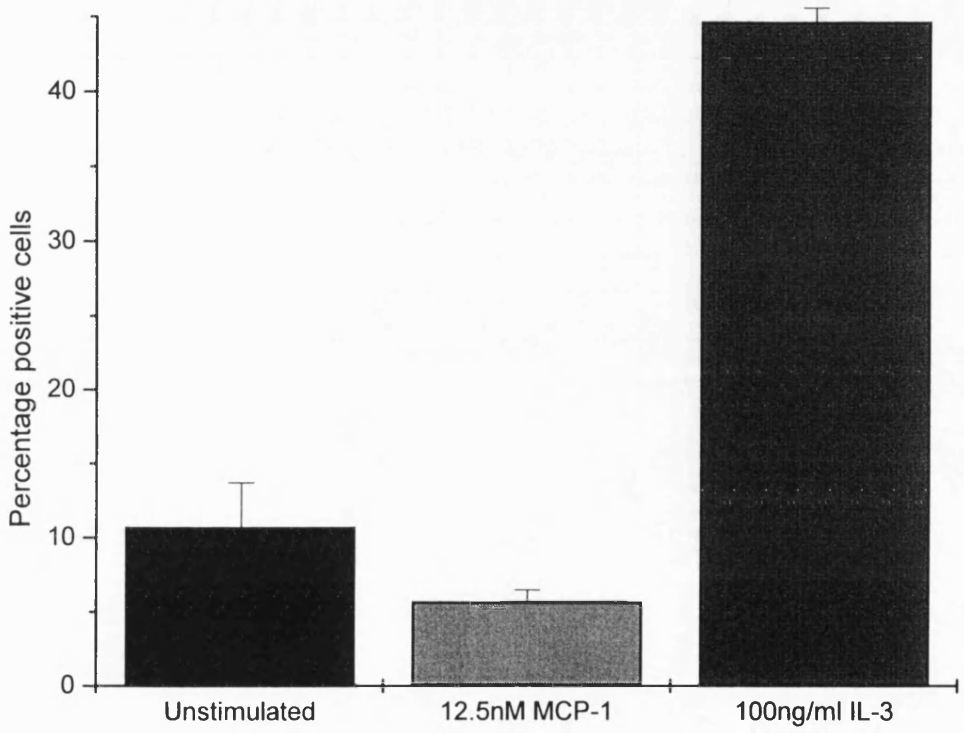


Figure 6.7 - Effects of MCP-1 on the upregulation of CD23 on human monocytes.
The PBMC's were prepared as given in section 2.2.1.3 and 2-5x10^6 cells/ml were plated out into Petri dishes and treated with either vehicle ( ), 12.5nM MCP-1 ( ) or 100ng/ml IL-3 ( ) for 44 hours. The cells were then labelled with anti-CD23-FITC antibody as given in section 2.2.14. The expression of CD23 on the CD14 positive monocytes was analysed by FACS. The data is representative of three separate experiments and is expressed as the percentage positive cells (data +/- s.d.).

6.5 Summary

1. MCP-1 induces a chemotactic response in both human monocytes and THP-1 cells. This response is dose-dependent with an EC_{50} value of around 1nM.
2. The expression of both CD11b and CD11c on human monocytes was greatly increased upon MCP-1 stimulation, in a dose-dependent manner. VLA-4 was not upregulated by MCP-1. 100nM wortmannin pre-treatment of the cells did not appear to have any marked effect on the expression of either CD11b or CD11c.
3. MCP-1 induced the production of superoxide by purified human monocytes, as detected by the dose-dependent reduction of ferricytochrome C. This response was similar to that induced by C5a but smaller than that observed following fMLP or PMA stimulation.
4. IL-3 induced a 4-fold increase in the expression of CD23 on human monocytes. In contrast, MCP-1 did not have any effect on the expression of this low affinity IgE receptor.
5. Although MCP-1-induced chemotaxis and superoxide release were detected, large donor variations and very small responses made investigations into the roles of PLC and PI 3-kinase in these functional responses very difficult. Also, as demonstrated in the adhesion molecule experiments, pre-treatment of the cells with pharmacological agents such as pertussis toxin was very difficult due to the ease with which human monocytes become activated.

SECTION 7 : DISCUSSION

7.1 The binding of MCP-1 to its receptors

The results presented in this thesis identified a single, high affinity group of MCP-1 receptors on THP-1 cells with a Kd of 4.71nM and 20 000 receptors per cell. These results are comparable with those already reported. The published Kd values for MCP-1 binding to THP-1 cells vary from 0.44nM to 25.7nM and the number of high affinity receptors per cell range from 5 000 to 18 000 (Van Riper *et al.* 1993) (Wang *et al.* 1993). Both of these reports identified a single receptor type on THP-1 cells, as demonstrated by a straight line on the Scatchard plot.

Receptor desensitisation is a phenomenon that has been observed by a number of G-protein -coupled receptors (Hausdorff *et al.* 1990) and it provides a method of observing which agonists bind to the same receptors. There are a number of possible explanations for receptor desensitisation, including receptor phosphorylation (Hausdorff *et al.* 1990), alteration of sensitivity of G-proteins to guanine nucleotides (Small *et al.* 1987) and receptor downregulation (Samanta *et al.* 1990). Interestingly, the IL-8 B receptor becomes phosphorylated upon the binding of MGSA and the receptor is subsequently degraded (Mueller *et al.* 1995). Also, chemokine receptor internalisation and subsequent degradation has already been reported for both IL-8 and MCP-1 receptors at 37°C following agonist binding (Samanta *et al.* 1990) (Wang *et al.* 1993). Therefore, it is likely that both receptor phosphorylation and receptor internalisation play a role in the MCP-1-induced desensitisation detected in THP-1 cells in this study.

In the results presented in this thesis, stimulation of THP-1 cells with various combinations of chemokines demonstrated heterologous desensitisation, with prior

stimulation of the cells with MCP-1 inhibiting the subsequent MIP-1α, RANTES, MCP-3 and IL-8 responses. In contrast, only MCP-3 could desensitise the MCP-1-induced calcium response. These results indicate the presence of at least two, if not three, separate MCP-1 receptors on THP-1 cells, one binding MCP-1 and MCP-3 only, one binding MCP-1, MIP-1α and RANTES and possibly a third which binds MCP-1 and IL-8. The first two receptors are most likely to be the CC CKR 2 and CC CKR 1 respectively, both of which have been identified on THP-1 cells (Charo *et al.* 1994) (Neote *et al.* 1993). The third is possibly a promiscuous receptor which binds both C-C and C-X-C chemokines. An example is the DARC, although the DARC has not yet been identified as a biologically active receptor (Horuk *et al.* 1996). Therefore, it is possible that there is another, as yet unidentified promiscuous chemokine receptor also present on THP-1 cells.

These results are in contrast to the desensitisation results published by Vaddi and Newton (Vaddi & Newton, 1994a). They studied the cross-desensitisation between MCP-1, RANTES and MIP-1α in THP-1 cells and found that only readdition of the same agonist could cause desensitisation, thus indicating that none of the chemokines applied interacted with the same receptor. Variations in the clone of THP-1 used and the culture conditions may explain the disparity between these results.

In contrast to the radioligand binding studies presented in this thesis, which identified a single receptor group, the calcium desensitisation results show the presence of more than one MCP-1 receptor type. The CC CKR 2 is a high affinity MCP-1 receptor and is the most likely candidate for the receptor identified in the radioligand binding study. The other receptors identified by the calcium desensitisation experiments, namely the CC CKR 1 and the promiscuous receptor, are likely to be low affinity MCP-1 receptors, which are not expressed at such a high level as the CC CKR 2 and are

not detected in the radioligand binding studies performed either in this study or in other published studies.

The binding data obtained with the CC CKR 2B transfected cells was also comparable to the published data. The Kd for MCP-1 binding to these cells was calculated from the Scatchard plot presented in this thesis to be 2.69nM with the number of receptors per cell calculated at 22 500. MCP-1 binding to the CC CKR 2B transfected into HEK 293 cells has been reported by other groups, although the Kd values obtained were slightly lower than observed in my study (0.26nM and 0.166nM) (Myers *et al.* 1995) (Franci *et al.* 1995). However, no B_{max} values have been published for these cells.

Although no radioligand binding was detected in the CC CKR 2A transfected cells, the data from this study using biotinylated MCP-1 demonstrated that the CC CKR 2A is expressed at the cell surface, but the pattern was very unlike that seen with either THP-1 cells or the CC CKR 2B transfected cells. The CC CKR 2A transfected cells fell into two categories, those expressing MCP-1 receptors and those not expressing MCP-1 receptors. This biphasic response might be explained by the following three factors; 1) the receptor may only be transiently expressed on the cell surface and thus only a percentage and not all of the cells express the receptor at any one time 2) an inadequate receptor transfection and 3) low affinity binding interactions. Addressing the first point, transient receptor expression could explain the two populations of cells detected, as not all of cells may express the receptor at any one time. This might also explain why no radioligand binding was detected. As only a percentage of the cells express the receptor, this population may not be large enough to be detected by the radioligand binding assay. The second point is unlikely as the clone used was chosen due to its high expression of CC CKR 2A mRNA and as the cells are maintained in G418 selection medium it is unlikely that a population of non-receptor expressing cells would develop. With regard to the low affinity binding interaction between the agonist

and its receptor, the equal shifts in the fluorescence detected in the THP-1 cells, the CC CKR 2B and the positive population of the CC CKR 2A transfected cells makes this an unlikely theory for the lack of radioligand binding and, again, would not adequately explain the presence of two cell populations.

No reports of MCP-1 binding to the CC CKR 2A transfected cells have, to date, been published. However, it is very interesting to note that there have been a number of radioligand binding studies performed using various chemokines and their receptors which have demonstrated results not representative of the biological responses. This was first demonstrated by Neote *et al.* with the CC CKR 1, the MIP-1α and RANTES receptor, in which the Kd values obtained with MCP-1 and MIP-1β were lower than for RANTES. However, a 10-fold excess of MCP-1 and MIP-1β were required to produce a calcium response and even then it was only 20% of the RANTES response (Neote *et al.* 1993). Similar anomalies have been observed by a number of groups during the characterisation of new members of the CC chemokine receptor group. For example, two independent groups have transfected CC CKR 3 into HEK 293 cells and, although they have found differences in the agonist specificity's, neither group can detect significant ligand binding by agonists which produce potent calcium responses (Combadiere *et al.* 1995) (Kitaura *et al.* 1996). Similar results have also been observed in HL60 cells transfected with the CC CKR 4 and in Chinese hamster ovary K1 (CHO K1) cells transfected with the proposed CC CKR 5 (Hoogewerf *et al.* 1996) (Samson *et al.* 1996). In both of these studies no significant radioligand binding could be detected in these cells using agonists which produced biological responses. These groups concluded that there must be either very low affinity binding interactions between the agonist and its receptor or inadequate receptor expression in the transfected cells.

Similar discrepancies between radioligand binding data and biological responses have been observed in the results presented in this thesis with the CC CKR 2A transfected cells. Although biological responses were detected in these cells, such as PI 3-kinase activation, no radioligand binding was observed. Interestingly, Charo presented some data at the 4th International Chemokine Symposium in which he showed that the CC CKR 2A was expressed within the transfected cells but was not translocated and subsequently expressed at the surface of the cells. He proposed that the alternatively spliced C-terminal tail prevented the appearance of the receptor at the cell surface. The biotinylated MCP-1 binding presented in this thesis has demonstrated that the CC CKR 2A is expressed at the surface of a small population of these cells.

7.2 Generation of calcium transients in response to MCP-1

7.2.1 MCP-1 induces a rise in $[Ca^{2+}]_i$ in THP-1 cells and human monocytes

The use of factors affecting both calcium influx and calcium mobilisation identified the source of calcium utilised in the MCP-1-induced $[Ca^{2+}]_i$ elevations in THP-1 cells. EGTA and the calcium influx inhibitor econazole (Mason *et al.* 1993) (Vostal & Fratantoni, 1993) had no effect on the peak height of the MCP-1-induced calcium transient, although EGTA did reduce the $T_{1/2}$ of the response. A possible explanation for the reduction in $T_{1/2}$ is that although the extracellular calcium does not play a role in the peak height of the calcium response, it may be involved in the refilling of the calcium stores and the maintenance of the calcium response. The lack of calcium influx in response to MCP-1 was confirmed by the lack of Mn^{2+} influx. 1mM Ni^{2+} did inhibit the MCP-1-induced calcium response but this concentration of Ni^{2+} has been reported to inhibit PLC activity (Azula *et al.* 1993) and so its effects as a selective calcium channel blocker may be confused with its PLC inhibitory activity. These results collectively indicate that there is no role for calcium influx in the MCP-1-induced calcium response in THP-1 cells.

The effects of the PKC inhibitor Ro31-8220-002 and the PKC activator PMA were also investigated in this thesis. These compounds were observed to potentiate and inhibit the calcium response in THP-1 cells respectively, indicating some form of negative feedback mechanism. Similar results have also been observed with PKC activators and inhibitors on thrombin-induced $[Ca^{2+}]_i$ elevations in platelets (Poll & Westwick, 1986) (Murphy & Westwick, 1992). Pre-treatment of platelets with PMA inhibited the thrombin-induced calcium response. However, administration of the PKC inhibitor Ro31-8220-002 to the platelets prior to thrombin stimulation had no effect on the peak height of the response but significantly increased the duration of the calcium response. PKC has been found to have two roles which directly affect the mobilisation of calcium from intracellular stores by IP_3. 1) PKC activates the IP_3 5-phosphatase which induces the breakdown of IP_3 to IP_2, thus reducing calcium mobilisation (Connolly *et al.* 1986). 2) PKC inhibits PLC thus preventing the generation of the calcium mobiliser IP_3 (Smith *et al.* 1987). These effects might explain the results obtained with the MCP-1 response and indicate a role for both PKC and PLC in the induction and maintenance of the MCP-1-induced calcium response in these cells. However, as well as having a direct effect on calcium mobilisation, PKC could also phosphorylate the IP_3 receptor itself or the CRAC channel, explaining the results obtained by some groups in which PKC inhibits capacitative calcium entry (Montero *et al.* 1993) (Petersen & Berridge, 1994). This may explain the results observed in platelets, in which the PKC inhibitor prolonged the calcium response, perhaps prolonging calcium influx. However, the lack of any detectable calcium influx in response to MCP-1 makes the latter effect of PKC less applicable to the MCP-1 response.

The role of PLC in the MCP-1-induced elevations in $[Ca^{2+}]_i$ was further confirmed in my study by the dose-dependent abrogation of the calcium response by the PLC inhibitor U73122.

However, the use of inhibitors to study the signal transduction pathways utilised by an agonist must always be interpreted cautiously, particularly in the case of U73122 which has been found to have effects other than inhibition of PLC (Alter *et al.* 1994) (Abayasekara & Flint, 1993). Therefore, the most accurate method of studying PLC activation is through the generation of IP_3. Results presented in this thesis demonstrated significant increases in IP_3 following MCP-1 stimulation. These increases preceded the calcium response proving the role of PLC in the induction of calcium transients by MCP-1.

The roles of calcium influx and calcium mobilisation in the MCP-1-induced elevations in $[Ca^{2+}]_i$ in THP-1 cells, observed in this thesis, are in stark contrast to those demonstrated by Sozzani *et al.* in human monocytes (Sozzani *et al.* 1993). This group first demonstrated in 1993 that the rise in intracellular calcium in human blood derived monocytes was dependent on the influx of calcium from the extracellular medium. This was deduced following the complete inhibition of the calcium transients by the calcium chelator EGTA and the calcium channel blocker Ni^{2+} as well as a significant dose-dependent quench of the fluorescence signal when Mn^{2+} was used as a surrogate calcium ion. They also investigated the effects of MCP-1 on the levels of IP_3 and found no agonist-induced increase in IP_3.

The main difference between my study and that carried out by Sozzani *et al.* was the time points at which the IP_3 levels were measured following MCP-1 stimulation. The first timepoint taken by Sozzani *et al.* during the IP_3 measurements was at 15 seconds which, in my study, was after the peak of the MCP-1-induced IP_3 response. Although

they performed the experiment in the presence of LiCl to try to prevent IP_3 metabolism, LiCl only inhibits IP phosphatase and inositol polyphosphate-1-phosphatase (Berridge & Irvine, 1989). Thus, although LiCl prevents inositol formation, a precursor lipid required for IP_3 generation, it does not inhibit IP_3 5-phosphatase which metabolises IP_3. Therefore, it is possible that the MCP-1-induced increase in IP_3 in human monocytes was missed.

Interestingly, Sozzani *et al.* extended their study of monocyte calcium transients in response to MCP-1 to include adherent monocytes and found that there was a significant role for calcium mobilisation as well as calcium influx (Bizzarri *et al.* 1995). The results from this latter study were very similar to the results obtained in my study with blood derived monocytes in suspension. The results presented in this thesis demonstrated that EGTA inhibited the calcium response but that this calcium influx was dependent on the initial calcium mobilisation, as indicated by the complete abrogation of the response by U73122. This demonstrates the possible activation of a capacitative calcium entry pathway in these monocytes by MCP-1, explaining why the influx is dependent upon the initial mobilisation of calcium.

Another member of the CC chemokine family, namely RANTES, has also been demonstrated to induce a calcium release activated calcium current. Bacon *et al.* observed that with very high concentrations of RANTES i.e. 1μM, a biphasic calcium response was obtained in human clonal T cells with the second phase being due to calcium influx (Bacon *et al.* 1995). Using patch clamp experiments they found that the properties of this phase were identical to those of the I_{CRAC} observed in Jurkat T cells following TCR activation. This is another demonstration of the link between calcium mobilisation and calcium influx following chemokine stimulation.

Unfortunately, due to the small numbers of monocytes that were obtained from each blood preparation, it was not possible to measure IP_3 levels following MCP-1 stimulation. It is also for this reason that the calcium traces using blood derived monocytes were more noisy.

The results from human monocytes presented in this thesis have demonstrated marked differences from those observed by Sozzani *et al.* (Sozzani *et al.* 1993). Differences in the monocyte preparation procedures might explain these variations in monocyte responses. With regard to this monocyte preparation question, it is interesting to note that the monocytes in this study were purified by adherence and the results obtained were similar to those observed for adherent monocytes by Sozzani and his co-workers (Bizzarri *et al.* 1995). In the previous study published by Sozzani *et al.,* the suspension monocytes were purified by Percoll gradient. Therefore, there is a possible effect of monocyte adherence which interferes with the calcium transients in response to MCP-1, perhaps involving integrin upregulation.

The results in this thesis have also detected a difference in the source of the calcium for the MCP-1-induced calcium transients in THP-1 cells and monocytes. This variation in the results between THP-1 cells and monocytes may be explained by the fact that THP-1 cells are a pro-monocytic cell line and may lack important signal transduction pathways vital for MCP-1-induced calcium influx. To examine this possibility, the biphasic response of ATP was observed in parallel with the MCP-1 response to confirm that THP-1 cells do possess an agonist induced calcium influx pathway.

The ATP-induced stimulation of THP-1 cells demonstrated in this thesis was demonstrated to induce a biphasic increase in $[Ca^{2+}]_i$. The factors affecting calcium influx, namely EGTA and econazole, almost completely abrogated the prolonged

calcium response, demonstrating calcium influx as a prerequisite for this secondary response. Calcium influx was further confirmed by the use of Mn^{2+} as a surrogate calcium ion, which demonstrated an ATP-induced dose-dependent Mn^{2+} influx response. The initial mobilisation of calcium in response to ATP was confirmed by the inhibition of the peak ATP response by the PLC inhibitor, U73122. The inhibition and potentiation of the initial peak of the ATP response by the PKC activator and inhibitor respectively, indicated a similar negative feedback effect of PKC on PLC as suggested for the MCP-1 response and thus further indicated the mobilisation of intracellular calcium.

Previous studies have demonstrated that, in macrophages, the initial phase of the ATP-induced calcium response is as a result of calcium mobilisation. The influx of calcium from the extracellular medium is responsible for the maintenance of the calcium response (Greenberg *et al.* 1988). These results relate well to the results presented in this thesis. The exact mechanism of calcium influx induced by purinergic receptor stimulation is not known (Alonso-Torre & Trautmann, 1993). It has been proposed to involve capacitative calcium entry although a role for receptor operated calcium channels cannot be ruled out. Interestingly, the observation in this thesis that inhibition of PLC reduced the subsequent calcium influx response points to an agonist-induced capacitative calcium entry.

Extracellular adenine nucleotides interact with multiple cell surface receptors and these recognise ATP, ADP and a number of synthetic analogues of these nucleotides (Dubyak & El-Moatassim, 1993). These receptors are all sub-types of the P_2 receptor class. These include the P_{2X} and P_{2Y} purinergic receptors, which were originally identified from studies on smooth muscle cells, the P_{2T} receptor, which is an ADP activated receptor on thrombocytes, the P_{2Z} purinergic receptor, which serves a non-selective pore-forming function and finally, the P_{2U} purinergic receptor, which is widely

distributed on a variety of cell types (Gordon, 1986). With regard to the purinergic receptors bound by ATP on THP-1 cells, Alonso-Torre and Trautman observed that it was the P_{2U} receptors on macrophages which induced both calcium mobilisation and calcium influx when bound by ATP (Alonso-Torre & Trautmann, 1993). Indeed, this was also demonstrated in THP-1 cells in this thesis using UTP. The P_{2U} receptor is the only purinergic receptor which is known to respond to UTP stimulation.

There have been mixed reports as to whether the ATP receptors are coupled to pertussis toxin-sensitive G-proteins (Sipma *et al.* 1994) (Lang *et al.* 1994) and from the results presented in this thesis, the lack of effect of pertussis toxin demonstrated that the P_{2U} receptor in THP-1 cells is not linked to either G_i or G_o G-proteins.

7.2.2 Disparate calcium signals generated by MCP-1 in the CC CKR 2A and 2B transfected cells

This study has already demonstrated that THP-1 cells possess more than one receptor which is activated by MCP-1. Therefore, it is beneficial to be able to study the high affinity MCP-1 receptors separately. The CC CKR 2A transfected cells demonstrated no detectable increase in $[Ca^{2+}]_i$. Interestingly, when the CC CKR 2A and 2B were first identified by Charo *et al.* they were injected into *Xenopus* oocytes and both were found to be equally effective at calcium efflux when stimulated by MCP-1 (Charo *et al.* 1994). However, there have been no further reports of CC CKR 2A inducing any calcium response.

In contrast, MCP-1 is a very potent inducer of calcium transients when added to HEK 293 cells expressing the CC CKR 2B. As yet, MCP-3 is the only other agonist active at this receptor. Results presented in this thesis demonstrated that MCP-3 induced a response only 25% of the response induced by an equivalent concentration of MCP-1.

In contrast, another group studying HEK 293 cells transfected with CC CKR 2B found MCP-3 to be equipotent (Franci *et al.* 1995).

The source of calcium in the MCP-1-induced intracellular calcium transients in these cells, as seen with the monocytes and THP-1 cells, is very controversial. In the results presented in this thesis both EGTA and Mn^{2+} demonstrated a role for calcium influx in the MCP-1 response in the CC CKR 2B transfectants. However, interestingly, the PLC inhibitor U73122 completely inhibited the MCP-1-induced calcium response, in a similar manner to that seen in monocytes, indicating a very close correlation between the calcium influx and calcium mobilisation, perhaps through capacitative calcium entry. These results are in contrast to those published by Myers *et al.*, who found that EGTA and Ni^{2+} had very little effect on the MCP-1-induced calcium flux in these cells except a small reduction in the $T_{1/2}$ (Myers *et al.* 1995).

As demonstrated with the THP-1 cells in this thesis, there was a time -dependent increase in IP_3 levels following MCP-1 stimulation of the CC CKR 2B transfectants, with the maximal increase preceding the rise in intracellular calcium. Again, this implicates PLC activation and the subsequent IP_3 generation in the MCP-1-induced calcium transients. The more rapid return of the IP_3 concentration to basal levels in the CC CKR 2B transfected cells compared to THP-1 cells could be due to the role of calcium influx in the transfected cells but not in THP-1 cells. Again, these results are in contrast to those observed by Myers *et al.* (Myers *et al.* 1995). They detected no increase in PtdIns turnover in response to MCP-1 in HEK cells expressing CC CKR 2B and suggested a novel mechanism for calcium mobilisation other than IP_3.

One very interesting observation from this thesis was that there was also a significant but very transient rise in the levels of IP_3 in the CC CKR 2A transfected cells following MCP-1 stimulation. However, these cells did not demonstrate any

increase in intracellular calcium. A possible explanation is that, as demonstrated by the binding of biotinylated MCP-1, only a small number of the cells express the receptor at any one time and it is not a large enough percentage to detect calcium responses on the fluorimeter. However, 10^7 cells are used for each point during the IP_3 measurement and thus small changes may be detected.

The effects of pertussis toxin on the MCP-1-induced calcium transients in the CC CKR 2B transfected cells were very similar in both my study and the study reported by Myers *et al.* (Myers *et al.* 1995). Even up to 250ng/ml pertussis toxin pre-treatment would only reduce the response to 20% of the control response. 100% inhibition was never observed. This is very interesting in light of a study carried out by Kuang *et al.* (Kuang *et al.* 1996). In this study they looked at the coupling of CC CKR 2A and 2B to α-subunits of the G_q class, which are insensitive to pertussis toxin. CC CKR 2B could couple to both Gα14 and Gα16 but CC CKR 2A could not. They also compared these two receptors to the CC CKR 1 and found the CC CKR 1 could only couple to Gα14. These α-subunits of the G_q class of G-proteins may be responsible for the residual pertussis toxin-insensitive calcium response seen in my study and by Myers *et al.* (Myers *et al.* 1995). The lack of a detectable calcium response in the CC CKR 2A transfectants meant that a comparison of the pertussis toxin sensitivity of CC CKR 2A and 2B could not be performed. Although the main expression of the Gα14, 15 and 16 is in hematopoetic cells (Wilkie *et al.* 1991), it may be that they are also present in these HEK 293 cells. However, it must be pointed out that in the THP-1 cells, the calcium response was completely inhibited by pertussis toxin. It is most likely that the high affinity MCP-1 receptors detected on THP-1 cells by the radioligand binding studies are of the CC CKR 2 group, which have already been identified on these cells (Charo *et al.* 1994). Therefore, the results obtained in these transfected cells may be due solely to the cells in which the receptors are transfected and not an effect of the receptor itself. It must be remembered that HEK 293 cells are epithelial cells and not

monocytic cells. This may mean that epithelial cells and monocytes do not possess identical signalling pathways.

A similar explanation may also apply with regard to the differences in the calcium source in the transfected cells, THP-1 cells and monocytes. It is feasible that, although ATP identified a calcium influx mechanism in THP-1 cells, the MCP-1 receptors may not be adequately coupled to these mechanisms. Thus, the results observed with the monocytes and CC CKR 2B transfected cells may provide a more relevant picture of calcium transients induced by MCP-1 due to the fact that they are mature cells. Adherence could also play a crucial role in the calcium response as monocytes, purified by adherence, and adherent HEK 293 cells transfected with the CC CKR 2B show similar responses, but suspension THP-1 cells do not. All these factors must be considered when analysing the mechanism of MCP-1-induced calcium fluxes.

7.2.3 Role for PLC and calcium in monocyte activation

A number of studies have been undertaken in the last few years to establish the role for the rise in intracellular calcium in MCP-1 stimulated monocytes. Locati *et al.* reported that MCP-1 stimulated the release of [^{3}H] arachidonic acid from pre-labeled monocytes and THP-1 cells (Locati *et al.* 1994). They found that this arachidonic acid release was completely abrogated by the presence of EGTA or Ni^{2+}, indicating a role for calcium influx. The same group also found that inhibition of calcium influx directly inhibited MCP-1-induced monocyte chemotaxis (Sozzani *et al.* 1993). However, the role for calcium influx in MCP-1-induced calcium transients and thus monocyte activation is debatable as demonstrated in the present study and in other published studies. As yet, no group has performed a detailed study of the role for PLC, IP_3 and calcium mobilisation in monocyte activation and functional responses. However, Vaddi and Newton observed that, in the presence of EGTA-acetoxymethyl ester (AM),

which chelates intracellular calcium, there was a 68% inhibition of the MCP-1-induced adhesion molecule upregulation (Vaddi & Newton, 1994b). Both intracellular and extracellular calcium had to be removed for complete inhibition, demonstrating a role for both calcium influx and calcium mobilisation in adhesion molecule upregulation. Although in the present study monocyte functional responses, such as chemotaxis and superoxide release, were observed, the donor variability and small signal to noise ratios made it very difficult to identify a role for calcium in these responses.

7.3 MCP-1-induced activation of PI 3-kinase

7.3.1 MCP-1 activates at least two PI 3-kinase isotypes in THP-1 cells

The activation of PI 3-kinase has been identified in a number of different systems in response to various agonists which activate both tyrosine kinase -coupled and G-protein -coupled receptors. In this study two separate methods of assaying PI 3-kinase in THP-1 cells were used, namely the accumulation of D-3 PtdIns lipids and an *in vitro* lipid kinase assay. Direct comparisons between the two separate assays are difficult since one is based on the measurement of PI 3-kinase products, such as PtdIns(3,4,5)P_3 in intact cells and the other depends on changes in PI 3-kinase activity that can be immunoprecipitated with a p85 antibody. Thus, in the intact cell assay, there may be other enzymes which may be contributing to the metabolism of the PI 3-kinase products. For example, the kinetics of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 generation in both the time-course and the dose-response studies show the same trends. In both of these studies it is the PtdIns(3,4,5)P_3 which appears only transiently and it is the PtdIns(3,4)P_2 which demonstrates a more sustained rise. It is possible that over time and at higher concentrations of MCP-1, the activation of a 5-phosphatase contributes to the generation and breakdown of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively. Although a number of experiments have been performed by other groups to try to establish the enzymes responsible for the

generation of these phospholipids (Stephens *et al.* 1991), the activation of a PtdIns(3,4,5)P_3 5-phosphatase cannot be ruled out in the present system. The activation of other enzymes involved in the generation and breakdown of the various phospholipids in this study is further confirmed in light of the fact that small changes were detected in PtdIns(3)P following MCP-1 stimulation of CC CKR 2A transfected cells. In other systems, the levels of PtdIns(3)P have been observed to remain unaltered upon agonist stimulation (Stephens *et al.* 1991). Again, the activation of other phosphatases may be important in the maintenance of this phospholipid. This latter observation may also be explained by the presence of a PtdIns specific PI 3-kinase activity, similar to the PtdIns-specific PI 3-kinase and the yeast Vps34 (Volinia *et al.* 1995) (Cárdenas *et al.* 1988). This is particularly interesting in light of the fact that only the CC CKR 2A transfectants show notable increases in PtdIns(3)P and not THP-1 cells or the CC CKR 2B transfectants.

However, taking into consideration the difficulties with direct comparisons between the two assay systems, there are a number of differences observed between the accumulation of PI 3-kinase products and the PI 3-kinase activity immunoprecipitated with the p85 antibody in THP-1 cells. 1) The time-course of activation appears to be very different, with PtdIns(3,4,5)P_3 appearing very rapidly but the activity of the immunoprecipitated PI 3-kinase only notably appearing at 1 minute and increasing with time. A similar difference is seen with the dose-response curves. It has already been reported that G-protein -coupled, PI 3-kinase γ can elicit the most rapid accumulation of PtdIns(3,4,5)P_3 for example in fMLP stimulated neutrophils (Stephens *et al.* 1993). The dose-response and time-course of PI 3-kinase product accumulation following MCP-1 stimulation is very rapid and may constitute the activation of PI 3-kinase γ. The p85 immunoprecipitates only isolate the classical PTK/SH2-coupled PI 3-kinase and not the distinct PI 3-kinase γ and the results presented in this thesis demonstrate that the time-course and dose-response curves for the activation of the

PTK/SH2 -coupled PI 3-kinase isoform are much slower than observed for the accumulation of the PI 3-kinase products. The disparate results obtained with the two assays presented in this thesis were made even more apparent by the use of both wortmannin and pertussis toxin. 2) Wortmannin had no effect on the generation of PI 3-kinase products when used at 100nM but potently inhibited the immunoprecipitated PI 3-kinase with an IC_{50} between 0.1 and 1nM. Stephens *et al.* reported that the ATP-induced stimulation of PI 3-kinase in U937 cells was due to the activation of a G-protein -coupled PI 3-kinase which was less sensitive to wortmannin (Stephens *et al.* 1994). This group further identified that the sensitivity to wortmannin also differed depending on the assay used. Wortmannin was 2.5-fold less active on the isolated G-protein -coupled PI 3-kinase but this increased to 6.5-fold when intact cells were studied. The IC_{50} for wortmannin on the G-protein -coupled PI 3-kinase in intact cells was approximately 130nM. Thus, 100nM wortmannin may not markedly inhibit the MCP-1-induced accumulation of PI 3-kinase products if the PI 3-kinase γ is the major PI 3-kinase activated in these cells. 3) The effects of pertussis toxin observed in this thesis on the two separate assays were very different to those of wortmannin. Pertussis toxin at 100ng/ml almost completely abrogated the generation of PI 3-kinase products but had no obvious effects on the immunoprecipitated PI 3-kinase. These results relate well to other published data, in which both the fMLP and ATP induced activation of the G-protein -coupled PI 3-kinase in neutrophils and U937 cells respectively were completely inhibited by 1.5μg/ml pertussis toxin pre-treatment (Stephens *et al.* 1993). Interestingly, fMLP stimulation of human neutrophils leads to the activation of both PI 3-kinase γ and PTK/SH2 -coupled PI 3-kinases in a pertussis toxin-sensitive manner (Stephens *et al.* 1993). However, this is in contrast to the PI 3-kinase activity immunoprecipitated in my study following MCP-1 stimulation of THP-1 cells, which was insensitive to pertussis toxin. Therefore, the results presented in this thesis have identified two separate PI 3-kinase activities, activated by MCP-1 in THP-1

cells, characterised by their time-course of activation and susceptibility to wortmannin and pertussis toxin.

A recent study found similar data when stimulating THP-1 cells with concanavalin A (Con A) (Matsuo *et al.* 1996). By monitoring PtdIns(3,4,5)P_3 accumulation, the agonist-induced increases in PtdIns(3,4,5)P_3 were completely abrogated by pertussis toxin and yet the immunoprecipitated PI 3-kinase activity was insensitive to pertussis toxin. This was in contrast to the observations of Stephens *et al.* in neutrophils, in which both PI 3-kinase γ and PTK/SH2 -coupled PI 3-kinases stimulated by fMLP were sensitive to pertussis toxin. As observed in my study the time-courses and dose-responses for the activation of the two PI 3-kinase activities by Con A in THP-1 cells were different. However, in contrast to the results presented in this thesis, the immunoprecipitated PI 3-kinase activity demonstrated more transient activity with much higher agonist concentrations being required than for the accumulation of PI 3-kinase phospholipid products. The major difference between the immunoprecipitates prepared by Matsuo *et al.* and those presented in this thesis is that Matsuo *et al.* assayed the PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates, but the results presented in this thesis are following anti-p85 immunoprecipitation (Matsuo *et al.* 1996). The disadvantage of using anti-phosphotyrosine immunoprecipitates is that there are a large number of tyrosine phosphorylated proteins, e.g. cytoskeletal proteins, which may constitutively bind to either PI 3-kinase or other lipid kinases, thus providing a less accurate picture of specific PI 3-kinase activation (Pawson & Schlessinger, 1993). Thus, only anti-p85 immunoprecipitates were analysed in this study.

Matsuo *et al.* concluded that the 'whole cell' assay afforded a much more accurate picture of the *in vivo* activation of this enzyme. They proposed that the immunoprecipitated PI 3-kinase activity did not contribute significantly to the 'total'

enzyme activity detected in the 'whole cell' system and may only be involved in 'local' PI 3-kinase activation (Matsuo *et al.* 1996). A similar conclusion was reached by another group studying the activation of both PI 3-kinase isotypes in fMLP stimulated neutrophils (Stephens *et al.* 1993). Although fMLP stimulated the PTK/SH2 -coupled PI 3-kinase, they concluded that this activity only made a small contribution to the overall production of PI 3-kinase products. The results presented in this thesis also show that the overall PI 3-kinase activity is most likely to be due to the G-protein -coupled PI 3-kinase γ, following the sensitivity of the D-3 PtdIns lipid accumulation to pertussis toxin. PI 3-kinase γ activation also explains the more rapid accumulation of D-3 phospholipids following the activation by MCP-1 as PI 3-kinase γ confers very rapid PtdIns(3,4,5)P_3 production (Stephens *et al.* 1993).

The PTK/SH2 -coupled PI 3-kinase is also activated by MCP-1, but similar to the conclusion reached by both Matsuo *et al.* and Stephens *et al.*, it is possible that this PTK/SH2 -coupled PI 3-kinase does not contribute significantly to the overall PtdIns(3,4,5)P_3 formation in intact cells, following the observation that the MCP-1-induced accumulation of D-3 phospholipids but not the MCP-1-induced increase in PI 3-kinase activity in p85 immunoprecipitates were sensitive to pertussis toxin. The observation that the PTK/SH2 -coupled PI 3-kinase activated by MCP-1 is insensitive to pertussis toxin indicates that it is not activated by a pertussis toxin-sensitive G-protein-coupled MCP-1 receptor. There are two possibilities, 1) there is a tyrosine kinase-coupled chemokine receptor and 2) the MCP-1 receptor which activates the PTK/SH2 -coupled PI 3-kinase may be coupled to a G-protein other than G_i or G_o which, through calcium-induced PKC activation, stimulates the PTK/SH2 -coupled PI 3-kinase, as proposed by Stephens *et al.* for fMLP stimulation of the PTK/SH2 -coupled PI 3-kinase (Stephens *et al.* 1993). It is interesting to note that a recent study by Okada *et al.* demonstrated that the PTK/SH2 -coupled PI 3-kinase in THP-1 cells can be stimulated by both tyrosine phosphorylated peptides and G$\beta\gamma$ subunits (Okada

et al. 1996). The combination of both phosphotyrosyl peptide and Gβγ subunit stimulation produced a synergistic effect, with the accumulation of PtdIns(3,4,5)P_3 being greater than the sum of the individual effects. Therefore, the activation of the PTK/SH2 -coupled PI 3-kinase through the proposed pertussis toxin-insensitive G-protein may be a direct effect of the G-protein sub-units on the PTK/SH2 -coupled PI 3-kinase. Although the MCP-1-induced calcium response in THP-1 cells was completely abrogated by pertussis toxin, in the light of the pertussis toxin-insensitive residual calcium response in CC CKR 2B transfected cells, it is possible that there is an MCP-1 receptor coupled to G-proteins other than G_i or G_o.

It is also possible that the PTK/SH2 -coupled PI 3-kinase, demonstrated to be activated by MCP-1 in this thesis, may have either an adaptor molecule or a serine kinase function. The p85 subunit has already been demonstrated to possess an adaptor function linking the insulin receptor to other binding proteins (Sung *et al.* 1994). Thus, it is possible that in MCP-1 activated cells, the PTK/SH2 -coupled PI 3-kinase functions as an adaptor molecule in preference to a 3-kinase. Also, the presence of the serine kinase tightly associated to the PI 3-kinase may also play an important role in MCP-1 signalling. Both of these functions might explain why this PTK/SH2 -coupled PI 3-kinase does not appear to be of primary importance in the MCP-1-induced accumulation of D-3 phospholipids.

Interestingly, only one other chemokine, to date, has been identified as activating PI 3-kinase. This is the RANTES stimulation of T lymphocytes and this activity was immunoprecipitated using a p85 antibody. It was sensitive to wortmannin but the effects of pertussis toxin were not investigated (Turner *et al.* 1995b).

7.3.2 Disparate PI 3-kinase activation by MCP-1 stimulation of CC CKR 2A and 2B transfected cells

The use of the two separate assay systems for PI 3-kinase activation exhibited very marked differences when using HEK 293 cells transfected with either the CC CKR 2A or CC CKR 2B. By monitoring the MCP-1-induced accumulation of D-3 PtdIns lipids, activation of the CC CKR 2A induced large, rapid increases in PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. The use of EGF as a positive control also produced increases in these products and these increases were comparable with those reported in the literature for EGF on PC12 cells (Soltoff & Cantley, 1996) (Carter & Downes, 1992). In contrast, the CC CKR 2B cells showed a very high basal level of PtdIns(3,4,5)P_3 which, in some cases, decreased upon MCP-1 stimulation. The latter phenomenon may have been due to the activation of a receptor-coupled 5-phosphatase because when PtdIns(3,4,5)P_3 decreased there was an increase in PtdIns(3,4)P_2. The possible release of MCP-1 by these epithelial cells was briefly investigated as this may have been a possible explanation for the increased basal levels of PtdIns(3,4,5)P_3. Northern blot analysis and enzyme linked immunosorbant assay (ELISA) were used to investigate the possible expression of MCP-1 RNA and MCP-1 protein release respectively, in these cells. Preliminary data from one experiment indicated that these cells do not express or release detectable amounts of MCP-1. The possible effects of MCP-1 on the variation in the proliferation rates of the CC CKR 2A and 2B transfectants were also investigated because PtdIns(3,4,5)P_3 may moderate a proliferative / mitogenic signal as demonstrated by PDGF and CD28 studies (Coughlin *et al.* 1989) (Ward *et al.* 1995). The lack of effect of pre-incubation with MCP-1 alone or in combination with MCP-1-antibody lead to the assumption that MCP-1 was not a contributing factor to the differing proliferation rates. Although wortmannin and pertussis toxin pre-treatment were not administered, the lack of effect of MCP-1 makes its mitogenic role unlikely. It is interesting to note that chemokines have been reported to mediate cellular proliferation. The C-X-C chemokine IL-8 has

been reported to inhibit the proliferation of vascular smooth muscle cells in a dose-dependent manner (Yue *et al.* 1994). In another report, MCP-1 was demonstrated to induce vascular smooth muscle cell proliferation (Xu *et al.* 1996), but in contrast, in another report, vascular smooth muscle cell proliferation was inhibited by MCP-1 (Ikeda *et al.* 1995). Thus, there are mixed reports as to the effects of chemokines on cellular proliferation.

The high PtdIns(3,4,5)P_3 levels observed in the CC CKR 2B transfectants may have been due to some sort of endogenous PI 3-kinase activity associated with the CC CKR 2B but not detected with the CC CKR 2A. Not even EGF produced a response in these CC CKR 2B transfected cells, indicating that PI 3-kinase may be maximally activated in these cells. Another possible explanation for this high basal level of PtdIns(3,4,5)P_3 may lie in the clonal differences in the transfected cells. Only one clone was made available and analysis of other clones may have provided slightly different results. Attempts were made to examine the effects of serum starvation on the levels of PtdIns(3,4,5)P_3, but unfortunately no conclusive results could be observed. Serum starvation of the cells for four hours had no effect on the basal PtdIns(3,4,5)P_3 levels in the CC CKR 2B transfected cells. Twelve hours serum starvation rendered the cells only 50% viable (as determined by trypan blue exclusion) and no MCP-1-induced increases in either CC CKR 2A or 2B transfected cells were detected.

In contrast to the results obtained by studying MCP-1-induced D-3 phospholipid accumulation in the receptor transfected cells, p85 immunoprecipitated PI 3-kinase activity was increased following MCP-1 stimulation in the CC CKR 2B transfectants but not the CC CKR 2A transfectants. The CC CKR 2A transfected cells showed no increase in PTK/SH2 -coupled PI 3-kinase activity following MCP-1 or EGF stimulation

but the CC CKR 2B transfected cells showed a dose-dependent increase in PTK/SH2 -coupled PI 3-kinase activity in response to both agonists.

Following the results with THP-1 cells it would be tempting to speculate that the whole cell assay detects mainly the G-protein -coupled PI 3-kinase γ and, of course, the immunoprecipitation technique only detects the PTK/SH2 -coupled PI 3-kinase. Therefore, it is possible that CC CKR 2A is coupled to PI 3-kinase γ and the CC CKR 2B is coupled to the PTK/SH2 -coupled PI 3-kinase. It is also interesting to note that, compared to both the CC CKR 2B transfectants and THP-1 cells, the CC CKR 2A transfected cells are the only group of cells examined in this study which demonstrated notable increases in PtdIns(3)P. Although this can be generated by PtdIns(3,4)P_2 breakdown, it is also possible that a PtdIns specific PI 3-kinase is activated by these receptors either uniquely or in parallel with the G-protein -coupled PI 3-kinase. The only difference between the CC CKR 2A and 2B is at the intracellular C-terminal domain. The CC CKR 2A has 14 extra amino acid residues at the C-terminus which are not displayed by the CC CKR 2B. It is possible that this difference leads to differential activation of signalling pathways by these receptors. The CC CKR 2B has already been demonstrated in this thesis to couple to pertussis toxin-insensitive pathway for a small part of the calcium response (see section 4.2.2.3). Therefore, it is possible that this pertussis toxin-insensitive response may also be involved in PTK/SH2 -coupled PI 3-kinase activation.

The THP-1 cells have been shown to possess both the type A and type B MCP-1 receptors (Charo *et al.* 1994). Therefore, from the data obtained using the receptor transfected cells it is possible that it is the CC CKR 2A that is responsible for the G-protein-coupled PI 3-kinase activity in THP-1 cells. Binding of MCP-1 to the CC CKR 2B may confer the PTK/SH2-coupled PI 3-kinase activity observed in THP-1 cells, although higher concentrations of MCP-1 were required for maximal PI 3-kinase

activation in the p85 immunoprecipitates in the THP-1 cells than were required for the maximal activation in the CC CKR 2B transfectants. There still also exists the difference in the PtdIns(3)P accumulation between the THP-1 cells and the CC CKR 2A transfected cells. Therefore, it is possible that THP-1 cells possess at least on other, as yet unidentified MCP-1 receptor.

7.3.3 MCP-1 induces the tyrosine phosphorylation of proteins in THP-1 cells and CC CKR 2B transfectants

The results presented in this thesis demonstrate that MCP-1 can phosphorylate tyrosine residues on three separate proteins of molecular weights 50, 80 and 120kDa. Although the characterisation of these proteins was not undertaken, it is possible to speculate about the identity of these proteins. For instance, the 120kDa protein may well be the focal adhesion associated kinase, FAK. FAK is a 125kDa protein which has been shown to be localised in areas of cell adhesion (Zachary & Rozengurt, 1992). Because of its localisation, FAK has been thought to be involved in regulation of cell migration in response to adhesion to extracellular matrix proteins. FAK becomes phosphorylated either upon activation of cells by external stimuli such bombesin (Leeb-Lundberg & Song, 1991) or PDGF (Rankin & Rozengurt, 1994) or by direct cell adhesion to extracellular matrix proteins (Guan *et al.* 1991). Integrin clustering on the cell surface also stimulates tyrosine phosphorylation of FAK (Schaller *et al.* 1992) Therefore, its role in MCP-1-induced activation of monocytes would be important in the regulation of adhesion and subsequent diapedesis. If indeed the p120 band is FAK, this protein could be involved in the initiation of firm adhesion of the monocytes to the endothelial layer by integrins prior to diapedesis. It is interesting to note that the chemokine RANTES has been demonstrated to phosphorylate FAK in a time-dependent manner in a human antigen-specific T cell clone (Bacon *et al.* 1996). However, in contrast to the rapid tyrosine phosphorylation

of the 120kDa band demonstrated in this thesis, RANTES induced a much slower phosphorylation of FAK with the maximal phosphorylation at 5 minutes. Variations in the receptors activated by MCP-1 and RANTES might explain the variations in the phosphorylation of the 120kDa band. Bacon *et al.* also observed the phosphorylation of paxillin, a 68kDa protein, previously observed to be tyrosine phosphorylated following the activation of integrins (Kornberg *et al.* 1992). However, no band corresponding to this molecular weight was detected in the blots presented in this thesis.

It was also considered that the 120kDa protein could be the accessory protein Cbl, originally identified as the cellular homologue of the transforming protein of murine Cas NS-1 retrovirus that induces pro-B, pre-B and myeloid tumours in mice (Langdon *et al.* 1989). Although Cbl has only really been demonstrated to be phosphorylated upon T cell, B cell and some cytokine receptor stimulation, there is evidence that PI 3-kinase associates with Cbl and that Cbl may communicate between PI 3-kinase and the FcγR-coupled src type PTK (Tanaka *et al.* 1995) (Fukazawa *et al.* 1995) (Matsuo *et al.* 1996). However, attempts to strip the blots developed with 4G10 and to reprobe them with an anti-cbl antibody, showed no bands corresponding to the 120kDa cbl protein.

Identification of the other 50 and 80kDa proteins is not so easy. It was possible that these proteins may be involved in the activation of the PTK/SH2 -coupled PI 3-kinase identified in this system. However, there was one discrepancy. The immunoprecipitated PI 3-kinase activity stimulated by MCP-1 was insensitive to pertussis toxin but the tyrosine phosphorylation of these proteins was inhibited by pertussis toxin pre-treatment. The activation of pertussis toxin -sensitive tyrosine kinase activity may indicate a role for PKC, similar to the protein tyrosine phosphorylation observed upon fMLP stimulation of neutrophils (Berkow & Dodson,

1990) (Huang *et al.* 1990). Agonist-induced increases in intracellular calcium and DAG formation may either activate PKC which phosphorylates and thus activates PTKs or, in the case of calcium, activate the PTK directly, leading to the tyrosine phosphorylation of various proteins.

Con A stimulated THP-1 cells not only lead to the activation of both PTK/SH2 -coupled PI 3-kinase and PI 3-kinase γ but also the time-dependent phosphorylation of a number of proteins (Matsuo *et al.* 1996). The tyrosine phosphorylation was maximal at 1 minute and then rapidly declined. Two of these proteins were of similar molecular weight to those observed in this thesis following MCP-1 stimulation of THP-1 cells. These two proteins were at approximately 120kDa and 80kDa. However, very little reference was made to the identity of these proteins, apart from to say that they had identified Lyn and GAP(Ras-p21 GTPase-activating protein)-associated p62. Neither of these proteins corresponded with the bands observed in the results presented in this thesis. Matsuo *et al.* proposed that the p120 band may have been the product of the proto-oncogene c-*cbl* but no evidence for this was presented (Matsuo *et al.* 1996).

The lack of effect of wortmannin on the MCP-1-induced tyrosine phosphorylation demonstrated in this thesis indicates that this tyrosine phosphorylation is either upstream of PI 3-kinase; or is mediated by the wortmannin resistant G-protein-coupled PI 3-kinase γ.

Due to the discrepancies in the sensitivity of tyrosine phosphorylation and immunoprecipitated PI 3-kinase activity to pertussis toxin, a different approach was taken to try to identify the phosphorylated proteins which activate the PTK/SH2 -coupled PI 3-kinase. This PI 3-kinase was immunoprecipitated using a p85α antibody and then tyrosine phosphorylated proteins were detected by Western blotting using an anti-phosphotyrosine antibody. Preliminary data showed that one band appeared,

with delayed kinetics, at approximately 55kDa. This band appeared to be distinct from the 50kDa band observed in the phosphotyrosine immunoprecipitated blots as both the time-course and the migration distances were different. This 55kDa protein was not detected on the initial phosphotyrosine blot of whole cell lysates but was detected when only the tyrosine phosphorylated proteins associated with the p85 subunit were studied. Interestingly, the kinetics of the phosphorylation of this protein matched the kinetics of the activation of the immunoprecipitated PI 3-kinase. Although the PI 3-kinase activity was detected at 60 seconds post MCP-1 stimulation, the tyrosine phosphorylation of the 55kDa protein did not notably appear until 120 seconds but the maximal PI 3-kinase activation and tyrosine phosphorylation both occurred at 300 seconds. It is possible that there are other, as yet unidentified protein(s) which are involved in the initiation of this PTK/SH2 -coupled PI 3-kinase activity. The earlier proposal that the 120kDa band may have been Cbl is even less likely following the inability to co-precipitate the 120kDa band with an anti-p85 antibody.

As to the possible identity of the 55kDa protein, it may be a non-receptor linked tyrosine kinase such as one of the src family tyrosine kinases. The role of src family tyrosine kinases, namely the T cell specific lck, in PI 3-kinase activation has already been identified in systems such as TCR activation of PI 3-kinase (Carrera *et al.* 1994) (Ward *et al.* 1996). Therefore, comparing the molecular weight and the expression pattern of the various src family tyrosine kinases, it is possible that the 55kDa protein could be one of the following src kinases, fgr (55kDa), lyn (56kDa), hck (59kDa) or fyn (59kDa) (review (Woodgett, 1994)). However, further identification needs to be performed using Western blotting techniques.

The preliminary protein phosphorylation results obtained following MCP-1 stimulation of CC CKR 2A or 2B transfected cells were very interesting. There was very little tyrosine phosphorylation detected in the CC CKR 2A transfected cells with possibly a

small phosphorylation of an 80kDa band. However, in contrast, the CC CKR 2B transfected cells showed phosphorylation of the three proteins detected in THP-1 cells. It appears that only CC CKR 2B is markedly coupled to tyrosine kinases. These results correlate well with the earlier hypothesis that only the CC CKR 2B is coupled to the PTK/SH2 -coupled PI 3-kinase.

Therefore, it would appear that the activation of protein tyrosine phosphorylation in the THP-1 cells by MCP-1 is through the CC CKR 2B, as the results in the CC CKR 2B transfectants and THP-1 cells are very similar. However, it is interesting to note that the time-course of phosphorylation of these proteins was slightly different in the two cell types and therefore, it is possible that the tyrosine phosphorylation in the THP-1 cells also involves an as yet unidentified MCP-1 receptor. However, it cannot be ruled out that these differences may be due solely to disparate signalling mechanisms present in the THP-1 cells and the transfected cells.

There are a number of G-protein-coupled receptors which, whilst lacking tyrosine kinase activity, have been shown to have associated tyrosine kinase activity upon receptor stimulation. These include receptors for bombesin (Zachary *et al.* 1991), vasopressin (Leeb-Lundberg & Song, 1991) and bradykinin (Hordijk *et al.* 1994). With respect to chemokines, very little data has been published on their ability to induce tyrosine phosphorylation. However, the C-X-C chemokine IL-8 has been observed to activate the serine/threonine kinase p42/p44 MAP kinase also known as extracellular signal regulated kinase (ERK)-2 (Jones *et al.* 1995). ERK-2 is activated by phosphorylation of tyrosine/threonine residues (Rossomando *et al.* 1991) (Nel *et al.* 1990) and is responsible for the regulation of several different proteins. Its role in the activation of cytoskeletal elements (Gotoh *et al.* 1991) has led to the proposal that ERK-2 is involved in chemokine-induced cellular responses. This is further confirmed by the time-dependent, pertussis toxin-sensitive activation of ERK-2 by IL-8 in Jurkat

cells transfected with either the IL-8 RA or RB (Jones *et al.* 1995). Another group has also identified the tyrosine phosphorylation of a p130 src substrate and a 70kDa protein following stimulation of a placental cell line transfected with the IL-8 RB by the C-X-C chemokine MGSA (Schraw & Richmond, 1995).

Apart from the report discussed previously, demonstrating RANTES-induced FAK phosphorylation (Bacon *et al.* 1996) only one other report has been published on C-C chemokine tyrosine kinase activation (Bacon *et al.* 1995). Bacon *et al.* observed that RANTES activation of human clonal T lymphocytes led to a biphasic calcium response and tyrosine phosphorylation of a number of different proteins. The latter was proposed as being responsible for the second phase of the calcium response, due to the inhibitory effects of tyrosine kinase inhibitors (Bacon *et al.* 1995). However, the concentration of RANTES required for these responses was 1μM, which is at least 100 times the concentration required for RANTES-induced chemotaxis in T cells (Schall *et al.* 1990).

The results from the PI 3-kinase data and the tyrosine phosphorylation data presented in this thesis, demonstrate the activation of both pertussis toxin-sensitive and pertussis toxin-insensitive pathways in THP-1 cells following MCP-1 stimulation. The identification of a 55kDa protein which co-precipitates with the p85 subunit of PI 3-kinase, suggests that the pertussis toxin-insensitive pathway is activated by a receptor associated with a non-receptor-coupled PTK, for example a src kinase. This provides more evidence for the earlier hypothesis that a tyrosine kinase-coupled MCP-1 receptor associates with the PTK/SH2 coupled PI 3-kinase and is responsible for its activation. As yet, no corroborative evidence for the presence of a pertussis toxin-insensitive G-protein-coupled MCP-1 receptor has been obtained.

Although CC CKR 2B has been proposed as the receptor on THP-1 cells responsible for the activation of the PTK/SH2-coupled PI 3-kinase and protein tyrosine phosphorylation, discrepancies in both the MCP-1 concentrations required for PTK/SH2-coupled PI 3-kinase activation and the time-courses of tyrosine phosphorylation indicate that THP-1 cells may possess an as yet unidentified MCP-1 receptor, possibly coupled to a src kinase. Although DARC has yet to be shown to possess biological activity (Horuk *et al.* 1996), it is not a G-protein coupled receptor and either it, or another receptor, may be important in MCP-1-induced PI 3-kinase activation. It is interesting to note that DARC, or a similar chemokine receptor was observed in the calcium desensitisation experiments.

7.3.4 Activation of potential downstream effectors of PI 3-kinase

The results presented in this thesis identified the phosphorylation of p70 S6 kinase in a time-dependent manner in response to MCP-1. However, the pattern of phosphorylation was not quite as expected. In studies which examined the phosphorylation of p70 S6 kinase, there is a single band which appears at 70kDa. Upon agonist stimulation, this phosphorylated protein has reduced gel mobility and the band to appears to move upwards (Kilgour *et al.* 1996) (Parry & Ward, 1996). The results presented in this thesis demonstrated a different picture. There were three bands which were present at 70 kDa in the unstimulated samples. The number of bands increased to a maximum of four bands by 15 minutes and then started to return to background levels by 30 minutes. Interestingly, published reports of experiments performed on purified p70 S6 kinase have demonstrated the presence of a number of p70 S6 kinase polypeptides using SDS-PAGE. The major polypeptide was at 70kDa which appeared with a ladder of slightly smaller polypeptides at 68 and 66kDa (Price *et al.* 1989) (review (Woodgett, 1994)). Another minor set of polypeptides migrated at 93/95kDa. These two isoforms are known as the αII and αI isoforms respectively. The only difference between the two is an amino terminal 23 residue peptide on the αI

isoform (Grove *et al.* 1991). This segment is very basic and causes the anomalously slow electrophoretic mobility despite its calculated molecular weight of 59kDa. Both the αI and αII peptides are recognised by the p70 S6 kinase antibody and feature in the blots obtained in my study. It appears to be the αII isoform that is phosphorylated but rather than demonstrating a straightforward band shift, there is an increase in the number of bands. It is most likely that MCP-1 induces the phosphorylation of the 70kDa band, as seen in the other reports, but the other two bands remain relatively unaffected. Different protein separation (i.e. the use of 10% or 15% resolving gels in SDS-PAGE) might explain the varying results of these 'band shift' assays.

Therefore, the activation of PI 3-kinase and the increased phosphorylation of p70 S6 kinase in response to MCP-1, demonstrated in this thesis, indicates that p70 S6 kinase may be a downstream target of PI 3-kinase in this system. p70 S6 kinase can regulate the entry of cells into cell cycle (Downward, 1994) and it is possible that the MCP-1-induced activation of p70 S6 kinase might be important in the eventual differentiation of monocytes into macrophages which occurs once the monocytes have entered into the tissue.

7.3.5 Role for PI 3-kinase and tyrosine phosphorylation in monocyte activation

There are a number of functional responses which have recently been connected to PI 3-kinase activation. Stimulation of THP-1 cells by Con A not only leads to the activation of two PI 3-kinase activities but also induces a respiratory burst (Matsuo *et al.* 1996). This respiratory burst is sensitive to pertussis toxin and is proposed as being activated by the G-protein -coupled PI 3-kinase γ. Hence, this is the functionally coupled isoform with respect to respiratory burst. The PTK/SH2 -coupled PI 3-kinase may be coupled to another function.

Interestingly, in another system which possesses both tyrosine kinase and G-protein -coupled PI 3-kinase activity, namely thrombin activation of platelets, it is the PTK/SH2 -coupled PI 3-kinase which has been implicated in functional responses (Zhang *et al.* 1996). Stimulation of platelets with thrombin promotes the conversion of platelet integrin $\alpha_{IIb}\beta_3$ into a fibrinogen binding form required for platelet aggregation. By studying the differential inhibition of the PI 3-kinases by wortmannin and comparing it to wortmannin inhibition of $\alpha_{IIb}\beta_3$ activation, it was concluded that the tyrosine kinase -coupled PI 3-kinase induces activation of this platelet integrin. It is interesting to note that, in this study, PI 3-kinase γ was demonstrated to be almost insensitive to PMA but the PTK/SH2 -coupled PI 3-kinase was potently stimulated by PMA. The use of PMA might help in dissecting out the relative roles of the PI 3-kinase isoforms in monocytes and monocytic cells lines.

Other functional responses associated with PI 3-kinase include respiratory burst and superoxide release from fMLP stimulated neutrophils (Arcaro & Wymann, 1993) (Vlahos *et al.* 1995) and RANTES stimulated eosinophils (Bourne *et al.* 1995) as well as RANTES induced actin polymerisation and chemotaxis in T lymphocytes (Turner *et al.* 1995a) (Turner *et al.* 1995b) (see table 1.7 in section 1.6.2.4).

Therefore, the role of PI 3-kinase in MCP-1 stimulated monocytes could be in superoxide release, actin polymerisation or adhesion molecule expression. Although preliminary data indicates that the adhesion molecule expression in this study was not inhibited by wortmannin it may be the G-protein -coupled PI 3-kinase which activates adhesion molecule expression which is much less sensitive to wortmannin. Unfortunately, it was not possible to observe the effects of both wortmannin and pertussis toxin on MCP-1-induced monocyte functional responses in this system. This was due to a number of reasons including donor variation, poor signal to noise ratios

and, in the case of adhesion molecules, problems encountered whilst pre-incubating the cells with pertussis toxin.

With regard to the role of tyrosine phosphorylation, Bacon *et al.* demonstrated that herbimycin A, a src family tyrosine kinase inhibitor, potently inhibited the pertussis toxin-insensitive calcium influx phase of the RANTES induced calcium response indicating a role for tyrosine kinases in calcium influx. RANTES induced T cell proliferation was also inhibited by herbimycin A but unaffected by pertussis toxin (Bacon *et al.* 1995). The role of tyrosine kinases is less likely to be important in MCP-1-induced calcium transients in THP-1 cells as pertussis toxin completely abrogates the response. It is possible, however, that it is more important in the CC CKR 2B transfected cells due to the pertussis toxin-insensitive element of the calcium response.

7.5 MCP-1-induced functional responses in human monocytes and THP-1 cells

This study has identified a number of functional responses induced by MCP-1 in human monocytes which include chemotaxis, adhesion molecule upregulation and superoxide release. The major problem encountered whilst studying these responses was the large donor variation and the relatively small responses. Also, the monocyte purification step was by plastic adherence, which makes it likely that the monocytes are activated at the start of the experiment, making any study of MCP-1-induced monocyte activation very difficult. Other methods of monocyte purification were tested, such as negative selection, but, unfortunately, the viability of the cells was much lower.

The results presented in this study demonstrated that MCP-1-induced a dose-dependent chemotactic response in both human monocytes and THP-1 cells. The EC_{50} for this response was between 0.1-1nM. The chemotactic response of human monocytes to MCP-1 has already been reported with EC_{50} values for this response varying from 0.1-0.5nM (Yoshimura & Leonard, 1990) (Sozzani *et al.* 1991). However, the MCP-1-induced chemotactic response in THP-1 cells has only been observed by some groups. Vaddi and Newton reported seeing no significant chemotactic response to MCP-1 in THP-1 cells (Vaddi & Newton, 1994a). In contrast, Ming Wang *et al.* found that THP-1 cells produced a dose-dependent chemotactic response to MCP-1 with an EC_{50} of around 1nM (Ming Wang *et al.* 1993). The results presented in this thesis compare well with the findings of Ming Wang *et al.*. The main difference between the present study and that of Wang *et al.* was the size of the chemotactic response. The chemotactic index in my study was approximately half of that observed by Ming Wang *et al.*. Variations in both experimental procedures and the clone of THP-1 cells used, might explain the different chemotactic responses observed with THP-1 cells.

The results presented in this thesis also demonstrated that MCP-1 dose-dependently upregulated both CD11b and CD11c expression on human monocytes. VLA-4 expression was not observed to increase upon MCP-1 stimulation. Similar results have also been reported by other groups. Jiang *et al.* observed that MCP-1 induced the upregulation of both CD11b and CD11c but there was no agonist induced upregulation of either CD11a or VLA-4 (Jiang *et al.* 1992). These results were confirmed and extended by Vaddi and Newton in 1994 (Vaddi & Newton, 1994b). Jiang *et al.* chose a 30 minute timepoint for their studies but Vaddi and Newton performed a time-course and observed that although there was a significant increase at 30 minutes, the maximum increase in both CD11b and CD11c was at four hours. The results presented in this thesis were obtained following a 30 minute incubation

with MCP-1 and the results obtained were very similar to those observed by Jiang *et al.*. Consistent in both the results presented in this thesis and those published by other groups is the maximal 2-fold increase in both CD11b and CD11c following approximately 30nM MCP-1 stimulation. Also consistent in all these studies was the higher basal level of CD11b compared to CD11c on human monocytes.

One of the main problems encountered when studying adhesion molecule expression on monocytes is the upregulation of these adhesion molecules by Ficoll during the monocyte purification process. Thus, by the time the agonist is administered, the adhesion molecule levels are already higher than expected. Therefore, in my study this was overcome by treating whole blood with MCP-1 and then lysing the red blood cells and separating the monocyte population by scatter properties and CD14 positive fluorescence on the FACS. However, this methodology creates its own problems, in as much as chemokines bind to the Duffy antigen on the red blood cells. Therefore, the shift is probably not as large as would be expected. It may be possible to block MCP-1 binding to red blood cells by adding another chemokine, such as IL-8, which will bind to the Duffy antigen but not to the monocytes and thus leave the MCP-1 free to induce monocyte adhesion molecule upregulation.

The MCP-1-induced upregulation of CD11b and CD11c but not VLA-4 following a 30 minute stimulation period was probably due to the intracellular location of these adhesion molecules. Miller *et al.* observed that CD11b and CD11c are mobilised from latent pools in monocytes whereas the upregulation of VLA-4, as well as the β_2 integrin CD11a, requires protein synthesis and longer time periods (Miller *et al.* 1987). However, it must be considered that, for all the adhesion molecules investigated in the present study, an increase in avidity of the adhesion molecules for their counter receptors may also play an important role in MCP-1-induced adhesion which would

not be detected as an upregulation. This can, however, be detected using antibodies to specific active sites of the adhesion molecules (Landis *et al.* 1993).

The results presented in this thesis also demonstrated the MCP-1-induced superoxide release from human monocytes. However, this response was only detected by monitoring the reduction of ferricytochrome C and not by the production of H_2O_2 in response to MCP-1. It is possible that the relatively low concentrations of superoxide produced by MCP-1 compared to the other agonists may make the detection of H_2O_2, which relies on the spontaneous conversion of superoxide to H_2O_2 by SOD present in the cells, more difficult to detect. However, by monitoring the direct production of superoxide using the reduction of ferricytochrome C, the small changes in superoxide production may be more readily detectable. It is interesting to note that there have been mixed reports as to the effectiveness of MCP-1 at inducing superoxide release from human monocytes. Leonard *et al.* reported detecting little or no release of superoxide in monocytes in response to MCP-1 (Leonard & Yoshimura, 1990). In contrast, Zachariae *et al.* and Rollins *et al.* both reported MCP-1-induced superoxide release from human monocytes (Zachariae *et al.* 1990) (Rollins *et al.* 1991). In a similar manner to the differences observed in the method of detection of the superoxide release by MCP-1 in this thesis, this might also explain the discrepancies between reports as to the effects of MCP-1 on monocyte superoxide release.

The other functional response that was investigated in monocytes stimulated with MCP-1 was the upregulation of the low affinity IgE receptor, CD23. Various cytokines such as IL-3, IL-4, GM-CSF and macrophage-colony stimulating factor (M-CSF) all upregulate CD23 expression on monocytes (Williams *et al.* 1992) (Alderson *et al.* 1992). Allergic diseases are characterised by the development of an IgE immune response to low concentrations of environmental allergens. The identification of MCP-1 in tissue from asthmatic patients suggests a role for MCP-1 in allergic diseases

(Sousa *et al.* 1994). The specific role of MCP-1 in asthma has not been well characterised and this study was initiated to determine whether the upregulation of the low affinity IgE receptor CD23 was involved. However, having chosen an MCP-1 concentration optimal for other functional responses and a timepoint optimal for the other cytokines no MCP-1 induced CD23 upregulation was observed, although IL-3 induced a response. Therefore, although a complete time-course and dose-response for MCP-1 should be performed to confirm this conclusion, MCP-1 dose not appear to upregulate CD23.

SECTION 8 : CONCLUSIONS

8.1 General conclusions

There are a number of conclusions which can be drawn from the results presented in this thesis.

1) MCP-1 binds to high affinity receptors on the surface of THP-1 cells and cells transfected with the CC CKR 2B as determined by Scatchard analysis. Although biotinylated MCP-1 binding to a population of the CC CKR 2A transfected cells was detected, it is possible that the receptors are only transiently expressed on a proportion of the cells at any one time.
2) MCP-1 induces a rise in intracellular calcium in THP-1 cells and CC CKR 2B transfected cells. In both cell types this involves the activation of PLC and the generation of IP_3. In the CC CKR 2B cells, however, this generation of IP_3 also leads to the activation of a calcium influx pathway, possibly through capacitative calcium entry. A similar dual calcium pathway was also detected in human monocytes. In contrast, the CC CKR 2A does not appear to be coupled to a calcium pathway as the transfected cells showed no obvious calcium response to MCP-1, although the increase in IP_3 indicates that the calcium response may be below the level of detection. Although the MCP-1-induced calcium responses in both THP-1 cells and CC CKR 2B transfectants were inhibited by pertussis toxin, the residual pertussis toxin-insensitive response in CC CKR 2B transfectants implicates a second pertussis toxin-insensitive pathway.
3) MCP-1 induces a dose- and time-dependent increase in PI 3-kinase products in THP-1 cells which is coupled to a G_i or G_o G-protein, as demonstrated by the almost complete abrogation of the response by pertussis toxin and the relative insensitivity of the response to wortmannin. The activation of the distinct PTK/SH2 -coupled PI 3-kinase, observed by p85 antibody immunoprecipitation, is unlikely to

play a major role in MCP-1-induced PI 3-kinase activation and is activated by either a pertussis toxin-insensitive G-protein or by distinct PTK -coupled MCP-1 receptor. The activation of a separate receptor linked to a non-receptor-coupled tyrosine kinase is further supported by the observation of a 55kDa tyrosine phosphorylated protein co-precipitating with the p85 PI 3-kinase sub-unit.

4) Assuming from the THP-1 results that accumulation of PI 3-kinase products is primarily as a result of G-protein -coupled PI 3-kinase activity and the activity in p85 immunoprecipitates can only be as a result of PTK/SH2 -coupled PI 3-kinase activity, then the CC CKR 2A is only coupled to PI 3-kinase γ and the CC CKR 2B is only coupled to the PTK/SH2 -linked PI 3-kinase. It is most likely the differential coupling of the alternatively spliced C-terminal tails of these two receptors that lead to the disparate signal transduction pathways.
5) MCP-1 induces tyrosine phosphorylation of three proteins in THP-1 cells and CC CKR 2B transfected cells. No notable tyrosine phosphorylation was observed in the CC CKR 2A transfected cells. The fact that only the CC CKR 2B and not the CC CKR 2A shows tyrosine phosphorylation further confirms the differential coupling to signal transduction pathways and the activation of tyrosine kinases by the CC CKR 2B. The MCP-1-induced tyrosine phosphorylation in the THP-1 cells was via a G-protein -coupled receptor due to its sensitivity to pertussis toxin. This is most likely to involve the increase in intracellular calcium and PKC.
6) MCP-1 activates a number of monocyte functional responses including chemotaxis, adhesion molecule upregulation and superoxide release. It does not appear that the adhesion molecule upregulation is via the wortmannin -sensitive PI 3-kinase.

8.2 Proposed pathways activated by MCP-1

The activation of monocytes by MCP-1 is potentially very important in understanding their role in inflammation. This study has identified the activation of three separate signal transduction pathways activated by MCP-1. However, when looking closely at

the individual receptor subtypes rather than at THP-1 cells some fundamental differences appear. Figure 8.1 shows both the proposed pathways activated in THP-1 cells, known to possess both CC CKR 2A and 2B, as well as those activated in the CC CKR 2A and 2B receptor transfected cells. In THP-1 cells, MCP-1 activates both a pertussis toxin -sensitive and a pertussis toxin-insensitive PI 3-kinase. Pertussis toxin also inhibits the MCP-1 induced calcium transient. These results indicate the role for G_i and G_o G-proteins in the activation of both PI 3-kinase and PLC activation. The PI 3-kinase not inhibited by pertussis toxin is separate from the other PI 3-kinase activated by MCP-1 and is not coupled to a pertussis toxin -sensitive G-protein.

By comparing the results seen in the THP-1 cells with the receptor transfected cells, it appears that only the CC CKR 2A is coupled to the G-protein -linked PI 3-kinase but not significantly to PLC and calcium transients. In contrast, the CC CKR 2B is coupled to PLC β, which is activated by pertussis toxin-sensitive and possibly a second, pertussis toxin-insensitive G-protein or to PLC γ via a PTK. Only the classical PTK/SH2 -coupled PI 3-kinase appears to be activated by the CC CKR 2B. The results observed in the THP-1 cells can be accounted for by the fact that both of these MCP-1 receptors are present in these cells. However, there are still differences between the THP-1 cells and the transfected cells, such as pertussis toxin sensitivity for the calcium responses, PtdIns(3)P generation and the disparate time-course and dose-responses for the PTK/SH2-coupled PI 3-kinase activation and tyrosine phosphorylation respectively, which may be due to other receptors present on the THP-1 cells.

This study has also highlighted the variations which occur not only between cell lines and primary cells but also differences arising from purification techniques and the cells used in transfection systems. Therefore, caution has to be taken when assessing and

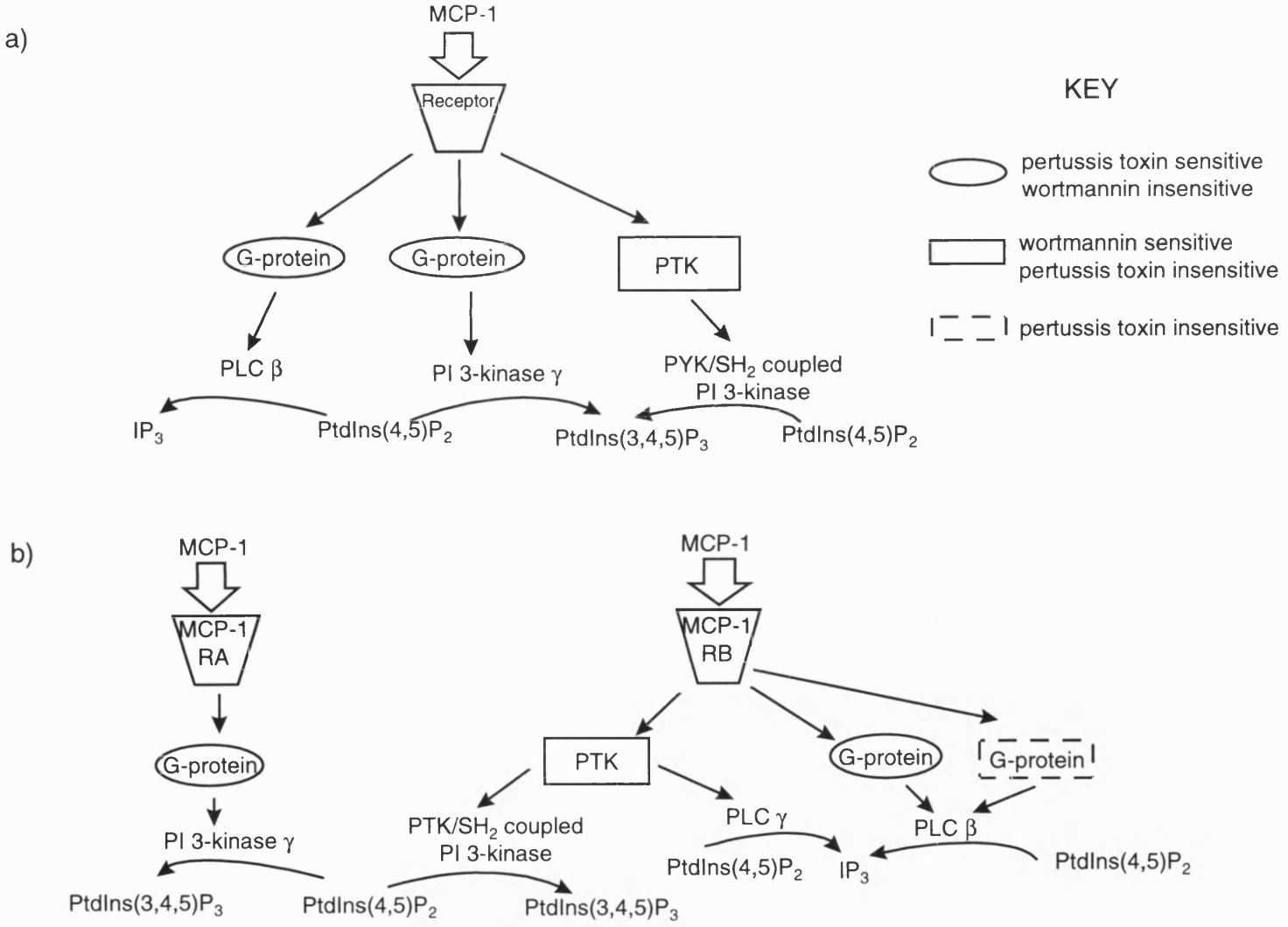


Figure 8.1 - Schematic diagram to represent the proposed pathways activated by MCP-1. a) the pathways activated in THP-1 cells and b) the pathways activated in CC CKR 2A and 2B transfected cells.

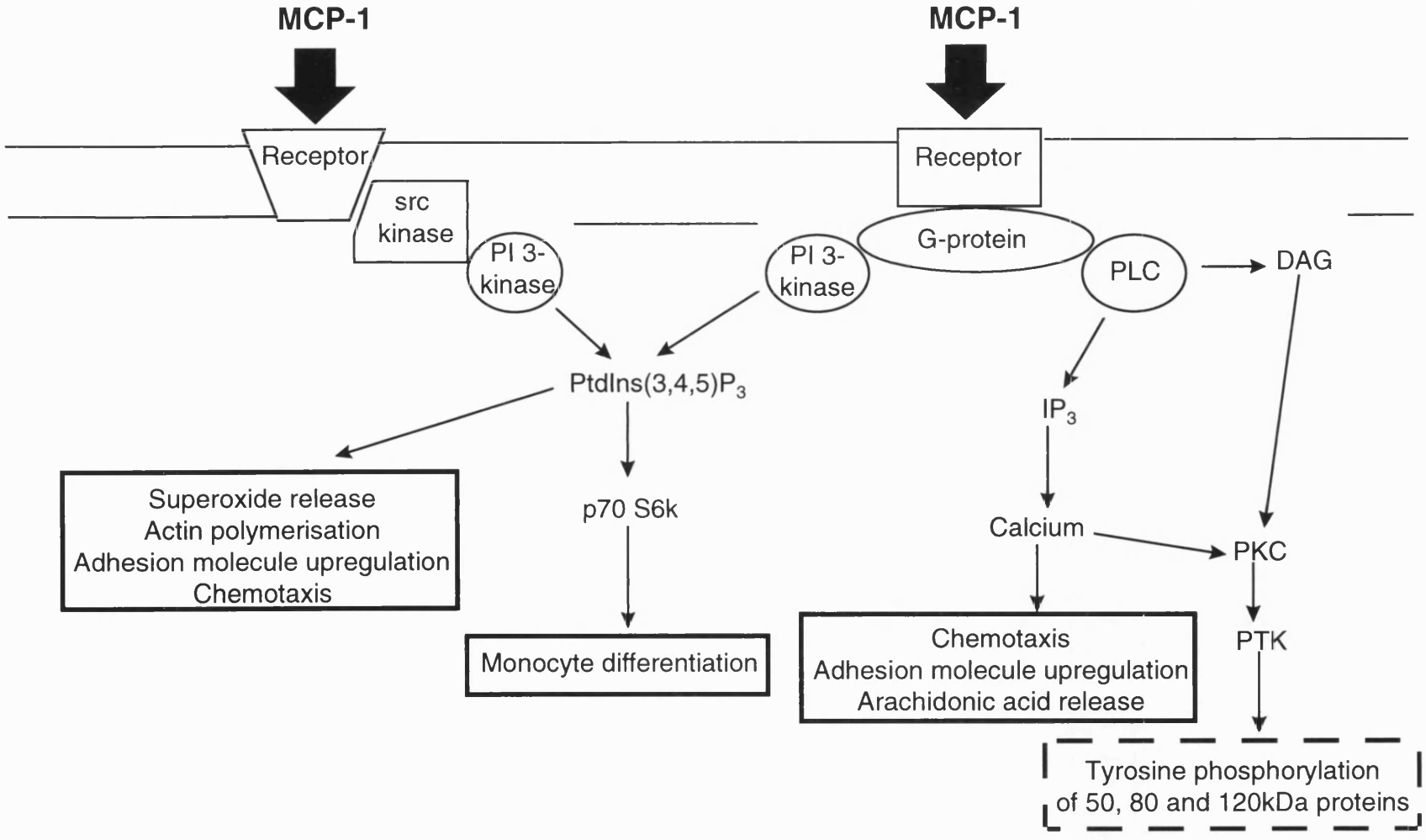


Figure 8.2 - Schematic diagram to show proposed roles for the signalling pathways activated by MCP-1. The intermediate siganlling molecules have not yet been identified and have been omitted for clarity. The bold boxes represent possible functional responses and the dashed boxes represent cellular responses.

comparing data as variations may be due to differential cellular responses rather than agonist induced responses.

From results obtained in this study and from published work by other groups, figure 8.2 shows a schematic diagram of the proposed roles of these signalling pathways in monocyte activation. Although some of the intermediate pathways have yet to be investigated, MCP-1 appears to activate three separate pathways. The majority of the responses are activated by pertussis toxin -sensitive G-proteins. However, MCP-1 also activates a PTK/SH2 -coupled PI 3-kinase which is insensitive to pertussis toxin and which is possibly activated by an as yet unidentified MCP-1 receptor which is coupled to a non-receptor PTK. The activation of PI 3-kinase is likely to be involved in both monocyte chemotaxis and other functional responses as well as their differentiation into tissue macrophages. The rise in intracellular calcium, be it either mobilisation, influx or a combination, may be important in similar pathways as PI 3-kinase, namely monocyte movement and functional responses. However, it may also play an important role in the phosphorylation of certain proteins by activation of PKC and subsequently PTKs. These phosphorylated proteins may help to modulate monocyte chemotaxis and functional responses by both direct mechanisms but also by activating other pathways which in turn modulate the responses.

Table 8.1 summarises all the responses which have been demonstrated to be activated by MCP-1 in this thesis. It is possible to correlate the activation of these responses with the events which take place *in vivo*. For example, the activation of PLC and the intracellular calcium rise is induced very rapidly by lower concentrations of MCP-1. Thus, these may be activated when the chemotactic gradient is very low. As the MCP-1 concentration increases down the gradient, the activation of pathways such as PI 3-kinase occurs possibly leading to the functional responses induced by higher concentrations of MCP-1 such as adhesion molecule upregulation. The

observation that the chemotactic response is induced by low concentrations of MCP-1 but adhesion molecules involved in the firm adhesion and diapedesis of monocytes are activated by higher concentrations of MCP-1 indicates the important role of an MCP-1 gradient in monocyte movement and activation. Functional responses such as superoxide release, although induced by lower concentrations of MCP-1 require much longer time-course. Therefore, it may be possible to apply the results obtained in this study to effects already observed *in vivo*.

There is a great deal of work left to be carried out before a clear picture as to the signalling pathways activated by MCP-1 and their role in the functional responses is obtained. However, the identification of MCP-1 in a number of inflammatory responses means that a full understanding of its mechanisms of action may be invaluable in therapeutic treatments in the future.

Table 8.1 - Summary of MCP-1-induced responses and the optimal concentrations and times. This table compares the concentrations and times required to stimulate the various responses detected after MCP-1 stimulation. The asterisks denote where only one concentration or timepoint was studied.

Response	Optimal [MCP-1]	Optimal time
IP_3 generation	*12.5nM	5 seconds
Intracellular calcium rise	12.5nM	10 seconds
G-protein -linked PI 3-kinase	60nM	30 seconds
Tyrosine phosphorylation	*180nM	30 seconds
p85 subunit phosphotyrosine protein interaction	*180nM	300 seconds
SH2/phosphotyrosine - linked PI 3-kinase	300nM	300 seconds
p70 S6 kinase phosphorylation	*180nM	15 minutes
Adhesion molecule upregulation	60nM	*30 minutes
Chemotaxis	3nM	*90 minutes
Superoxide release	12.5nM	120 minutes

8.3 Future directions

Although this study has gone part of the way to identifying the signalling pathways activated by MCP-1, there are a number of further studies that could be carried out to further characterise the MCP-1-induced activation of monocytes and monocytic cell lines.

1) To identify the possible transient nature of the expression of the CC CKR 2A receptor, the cells which are observed to bind MCP-1 using biotinylated MCP-1 could be sorted from the cells not expressing the receptor and then cultured. The cellular distribution of the receptors could then be monitored with time to see if a similar heterogeneity of receptor expression develops.
2) More investigation into the possible activation of PLC in the CC CKR 2A transfectants following MCP-1 stimulation, again by monitoring the possible increases in $[Ca^{2+}]_i$ in these cells following MCP-1 stimulation of the FACS. Any cells which may respond to MCP-1 by demonstrating an increase in $[Ca^{2+}]_i$ could be sorted from the non-responding cells and the possible changes in the cells which respond to MCP-1 with time could be studied.
3) Further investigation into the pertussis toxin-insensitive part of the MCP-1-induced calcium response in CC CKR 2B cells using PTK inhibitors may provide more evidence as to the possible coupling of the CC CKR 2B to tyrosine kinases. Following on from this, it would be useful to determine the relative expression of all the different Gα subunits of G-proteins in the HEK 293 cells as well as in THP-1 cells and human monocytes to further investigate the effects demonstrated by Kuang *et al.*. This could be achieved if antibodies to these G-protein subunits were available.
4) Further characterisation of the disparate PI 3-kinase activities stimulated by MCP-1 in the CC CKR 2A and 2B transfected cells using the pharmacological agents pertussis toxin and wortmannin. This would be particularly useful in trying to further elucidate the cause of the very high basal levels of PtdIns(3,4,5)P_3 in the CC CKR

2B cells. It would also further confirm the proposed variation in PI 3-kinase activities which are coupled to each of these receptors i.e. the CC CKR 2A is coupled to PI 3-kinase γ and the CC CKR 2B is coupled to the PTK/SH2 -coupled PI 3-kinase.

5) Further investigation is required into the proteins tyrosine phosphorylated by MCP-1 in THP-1 cells as well as CC CKR 2A and 2B transfected cells. This includes identifying the 120, 80, 55 and 50kDa proteins in THP-1 cells as well as studying the possible co-precipitation of the 55kDa protein with the p85 subunit of PI 3-kinase in CC CKR 2A and 2B transfectants. This would possibly provide further evidence for the diversity of the signalling pathways activated by MCP-1 binding to these receptors. Also, the effects of pertussis toxin and wortmannin on the phosphorylation of the 55kDa protein would possibly confirm the role of this protein in the activation of the pertussis toxin-insensitive PTK/SH2 -coupled PI-3 kinase. Also, the possibility that other proteins might also be required for the activation of the PTK/SH2 -coupled PI 3-kinase activation due to the slight delay in the appearance of the 55kDa band compared to the immunoprecipitated PI 3-kinase activity needs to be further investigated.
6) The expression of active and dominant negative p85 and p110 mutants may provide vital clues as to the role of PI 3-kinase in monocyte function and differentiation as well as the role of PI 3-kinase in MCP-1-induced responses.
7) To further characterise the phosphorylation of p70 S6 kinase by MCP-1 using both pertussis toxin and wortmannin and to try to determine whether this is a downstream effector of MCP-1-induced PI 3-kinase activation. PMA could be used as a positive control for p70 S6 kinase activation.
8) The identity of the proposed PTK -coupled MCP-1 receptor and/or the MCP-1 receptor coupled to a pertussis toxin-insensitive G-protein must be identified. Characterisation of the DARC receptor as well as attempting to clone other MCP-1

receptors which may be present in THP-1 cells would be very useful in the identification of this receptor.

9) The role of both PLC and the PI 3-kinase isoforms in monocyte functional responses is paramount in further characterising the MCP-1-induced activation of monocytes.

APPENDICES

Appendix 1

Cell culture medium

THP-1

RPMI 1640 with 25mM Hepes

2mM glutamine

100ug/ml penicillin

100units/ml streptomycin

10% FCS (v/v)

HEK 293 cells

MEM

0.1mM MEM non-essential amino acids

2mM glutamine

1mM sodium pyruvate

800μg/ml Geneticin (G418) (untransfected cells were cultured in the absence of G418)

10% FCS (v/v)

Appendix 2

Radioligand binding assay

Binding buffer

50mM Hepes

1mM $CaCl_2$

5mM $MgCl_2$

0.5% BSA (w/v)

pH adjusted to 7.2

Appendix 3

Preparation of cell lysates

Lysis buffer

100mM NaCl

20mM Tris

10mM NaF

10mM iodoacetamide

pH adjusted to 7.4

1% NP40 (v/v)

The following protease/phosphatase inhibitors were added immediately prior to use

1mM sodium orthovanadate

1μg/ml leupeptin

1μg/ml pepstatin

10mg/ml β-glycerophosphate

1mM PMSF

Appendix 4

***In vitro* lipid kinase assay**

Kinase buffer

5mM $MgCl_2$

0.25mM EDTA

20mM Hepes

pH adjusted to pH 7.4

Appendix 5

SDS-PAGE and Western blotting reagents

Sample buffer

2% SDS (w/v)

2% glycerol (v/v)

162mM Tris(hydroxymethyl)aminomethane (Tris) (pH 6.8)

9% 2-mercaptoethanol (v/v)

1mg bromophenol blue

Make up to correct volume with distilled water

Running buffer

25mM Tris base

192mM glycine

0.1% SDS (w/v)

pH must be above 8.3

Transfer buffer

39mM glycine

48mM Tris base

0.0375% SDS (w/v)

20% methanol (v/v)

Tris buffered saline (TBS)

20mM Tris base

2mM NaCl

adjust pH to 7.5

For TBS + NP40 0.05% NP40 (v/v) is added

Blocking buffer

5% BSA (w/v)

1% ovalbumin (w/v)

0.05% azide (w/v)

Make up to final volume in TBS

Coomassie blue stain

0.25% Coomassie blue (w/v)

45.4% methanol (v/v)

9.2% glacial acetic acid (v/v)

Make up to final volume in H_2O

Destain solution

45.4% methanol (v/v)

9.2% glacial acetic acid (v/v)

Make up to final volume in H_2O

Table A5 - Recipes for various percentage SDS-gels

(Resolving gel - 5 ml is sufficient for 1 mini gel and 40 ml is sufficient for 1 large gel

Stacking gel - 1 ml is sufficient for 1 mini gel and 8 ml is sufficient for 1 large gel).

Solutions	Resolving gels			Stacking gel
	7.5% (5 ml)	10% (5 ml)	15% (40ml)	5% (1 ml [8 ml])
H_2O	2.35	2	9.2	0.68 [5.5]
30% Acrylamide mix (w/v)	1.25	1.7	20	0.17 [1.3]
1M Tris (pH 8.8) {1M Tris (pH 6.8) for stacking gel}	1.3	1.3	10	0.13 [1]
10% SDS (w/v)	0.05	0.05	0.4	0.01 [0.08]
10% ammonium persulphate (APS) (w/v) - made up immediately prior to use	0.05	0.05	0.4	0.01 [0.08]
N,N,N',N'-tetramethylethylene-diamine (TEMED)	0.003	0.002	0.016	0.001 [0.008]

Appendix 6

Scopoletin assay for H_2O_2

Reaction mixture

69μg/ml scopoletin

15mM sodium azide

1mg/ml Horseradish peroxidase

2mM Ca^{2+}

2mM Mg^{2+}

Made up to final volume in HBSS without Ca^{2+} and Mg^{2+}

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